An investigation into diet and economy in ancient Mongolia through multiple biomolecular datasets

Dissertation

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1. Introduction

Studying the dietary diversity of past populations has the power to reveal how varied environments, population dynamics, and cultural practices have influenced human subsistence patterns. Expanding our understanding of the ways in which populations responded to environmental and political changes in the past can offer insights into the development of modern responses to similar changes. This is particularly relevant in Mongolia, an area often characterized as homogenous terrain occupied by specialized pastoralists, from prehistory to the present day; which resides within an environment increasingly threatened by climate change, pollution, and the demands of an extensively capitalist world system. However, due to a lack of solid stratigraphy, an overreliance on historical documentation, and limited application of archaeological science methodologies, our understanding of dietary and economic diversity in this part of the world has remained remarkably lacking, despite popular imaginations of Mongolian subsistence and mobility [1–4]. In this thesis, I apply stable isotope analysis and proteomic analysis to archaeological samples from Mongolia, spanning between Neolithic and the Mongol Empire periods (*ca*. 3000 BCE - 1400 CE) in order to determine the arrival of ruminant dairying on the eastern steppe, the origins of horse milk traditions, and the incorporation of grain agriculture into Mongolia's early economies. The three resulting studies of Mongolia's varied ancient and historic dietary traditions, across diverse environmental zones, offer a unique window into how eastern steppe populations subsisted through major ecological and political transitions.

Dairy pastoralism is a common global subsistence strategy that is especially prevalent across the Eurasian steppe and Mongolia today, however the date of emergence and the varied histories of this practice remain unclear. Economic and subsistence strategies in ancient Mongolia have been challenging to uncover, largely due to the paucity of excavated domestic sites prior to the Iron Age (ca. 800 BCE). Nevertheless, while occupation sites are seldom excavated, the prehistoric archaeological landscape is dominated by ritual stone monuments that often contain human burials. The recent rise and combination of a diversity of biomolecular methods that can reveal direct insights into foods consumed by an individual, mean that it is possible to draw out insights into changes in subsistence from funerary contexts.

To identify trends in dairying this thesis contains a study combining ancient protein analysis of human dental calculus in tandem with ancient DNA (aDNA) analysis of individuals from a Late Bronze Age (*c*. 1300 BCE) site in northern Mongolia (Manuscript 1) and an ancient protein analysis of an extended sample set of individuals spanning multiple time periods (3000 BCE – 1400 CE) and regions across Mongolia (Manuscript 2). To discover dietary trends related to C_3 (wheat, barley) and C_4 (millet, maize) grain consumption, we include a carbon and nitrogen stable isotope analysis of human bone collagen and carbon stable isotope analysis of dental enamel bioapatite (Manuscript 3). The use of these two methods allows us to investigate dairy intake through the identification of taxa-specific milk peptides in concert with C_3 and C_4 plant intake through stable carbon isotope ratios. Through the multi-proxy datasets produced in these studies, we create a timeline of changing subsistence practices and detail the earliest direct evidence for the consumption of dairy on the Eastern Eurasian Steppe (ca. 3000 BCE), explore the incorporation of horse milk into diets in the Late Bronze Age (ca.1200 BCE), and examine the diversification of diets during the Xiongnu and Mongol Empires (ca. 200 BCE - 1400 CE). Our results illustrate how early pastoral populations transitioned into diverse, imperial agro-pastoral dietary economies catalysed by the incorporation of horses, and horse riding, into existing herding practices.

1.1 *The origins and spread of dairying*

Today, worldwide milk production forecasts for 2019 are over 850 millions tons, as the consumption of milk and dairy products are an essential part of global subsistence strategies. The exact total differs from country to country, with individual consumption varying between 10 and 300 kg annually [5]. The initial suggestion for the origin of the dairying, among other secondary products (wool, traction), was put forth by Sherratt in the early 1980's [6]. The idea was that dairying did not begin until the Chalcolithic, and that it was the incorporation of these secondary products that enabled populations to become the larger, more complex political systems of the Bronze Age [6–8]. While many of Sherratt's ideas have been validated by subsequent research, testing of this hypothesis through zooarchaeological and lipid analysis of ceramic vessels has shown that dairying practices appear to have begun earlier. Evidence of dairy fats in ceramics are present shortly after, if not concurrently, with the first domestication of ruminant animals (sheep, goat, cattle) [8–12]. This incorporation of dairying practices into dietary systems resulted in a multitude of observable changes in the archaeological record. For example, optimization of dairy production, i.e. increasing milk production through retaining

milk-producing females to old age and killing males at a young age results in alterations in the ratio of females to males in herd composition, and animal age at death profiles [6,12].

Evidence for past dairying can be identified through the presence of dairy processing and storage materials, as well as through slaughter patterns of dairy animals for optimal milk exploitation [13]. For example, protein and lipid (fatty acid) evidence has shown the presence of milk (among other dietary materials) in ceramics in early Anatolia at (ca. 6500 BC) [10,14]. The movement of dairy practices northwards into Europe has been demonstrated through additional lipid evidence for ruminant dairy products from Neolithic sites in modern day Poland, where dairy fats were present in sieve-like ceramics with holes, suggestive of cheese making vessels [11,12]. The use of dairy in Europe is well-documented and has been extensively published [11,15–18], and while a few recent studies have touched on on the eastern spread of dairying into Asia, much more work is needed for a fully nuanced understanding of this dispersal. So far, a few published studies demonstrate the presence of dairy animals and practices in the central and eastern steppe through archaeological, lipid, and protein research [19–21] (Figure 1). Zooarchaeological evidence that suggests mortality profiles of zooarchaeological assemblages associated with dairy pastoralism have been found in southern Russia and Kazakhstan [19,22] indicating an eastward migration of herding practices. aDNA research documents the migrations of Bronze Age western steppe populations eastward, reaching as far as the Altai Mountains [23–25]; however, these studies suffer from a lack of contemporaneous samples feast of the Altai, and these migrations may have travelled further east into Mongolia.

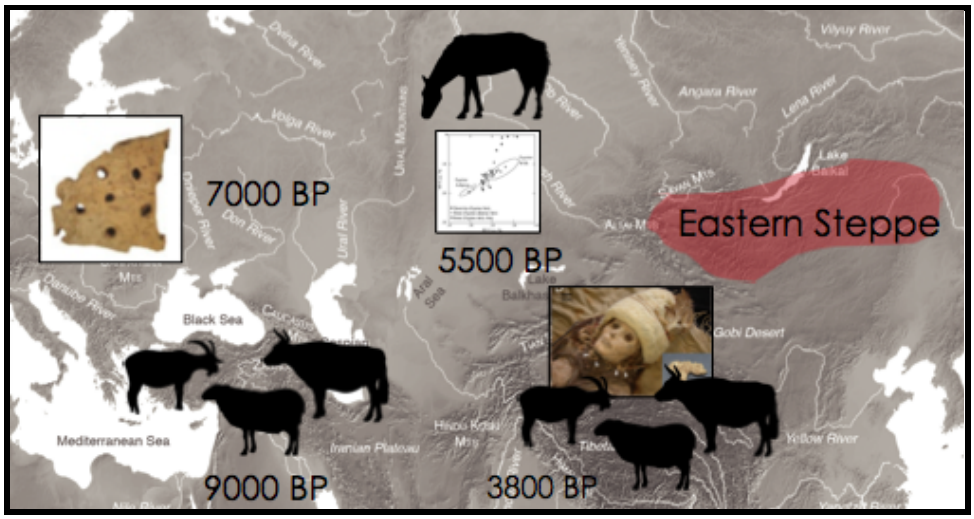

Figure 1: Examples of lipid and proteomic evidence for early dairying on the Eurasian Steppe (Craig et al. 2011; Evershed et al. 2008; Salque et al. 2013; Xie et al. 2016; Yang et al. 2014; Outram et al., 2009).

1.2 *The antiquity of dairying in Mongolia*

In contemporary Mongolia, dairy pastoralism is practiced by approximately 40% of the population living outside of the urban capital of Ulaanbaatar [28]. Depending on the environment and climate, seven different animal species are commonly milked in Mongolia: sheep, goat, cow, yak, reindeer, camel and horse (Figure 2). From the milk of these species many different dairy products are produced including fresh and fermented milk, yogurt, butter, and a diversity of cheeses and dried consumables [29]. In addition to caloric intake, milk consists of between 80-90% water [30], providing a source of vital hydration on the arid steppe. Dairying is regarded as a long-standing subsistence practice, and is believed to have extended far into the past, however, it remains unclear when or where the first animals were used for dairying on the Eastern Steppe.

Figure 2. Dairy animals of modern Mongolia: sheep and goat, yak, cow, camel, reindeer and horse.
Photo acknowledgements: Heirloom Microbes project [https://www.shh.mpg.de/349696/heirloom-microbes]

Mongolia's history is marked by imperial periods, such as the Mongol (1200 - 1400 CE) and Xiongnu Empires (ca. 200 BCE – 200 CE), and preceding prehistoric populations of the Early Iron Age (800 - 300 BCE), the Bronze Age (3000 - 800 BCE), and Eneolithic and Neolithic periods (prior to 3000 BCE). The Mongol and Xiongnu Empires have been colloquially understood as polities of hostile, horseback raiders that lacked the complexity of other well-known global imperial structures [31,32]. Nonetheless, as demonstrated in this thesis, both empires relied on diverse, multi-resource dietary economies including animals (milk, meat, transport, leather), crops, and imported foods [33–35]. Prior to the Xiongnu period, in the Early Iron Age, Bronze Age, and Neolithic periods, little is known about subsistence strategies due to a lack of historical evidence and the sparsity of excavated occupation sites [36,37].

There is ample historic evidence for dairy consumption from Mongol period texts, where specific mentions of ruminant and horse milk consumption are common. One such example is the "Secret Histories of the Mongols", translated into English by DeRachewiltz [38]. This written work is the first book known to have been composed by Mongols in the Mongolian language, and it details the life and rise of Chinggis Khan. Born as Temujin, a young boy from a rural pastoral family, Chinggis Khan grew up to unite disparate tribes from across the region into the Mongol Empire. "The Secret Histories of the Mongols" [38] repeatedly details the social importance of fermented horse milk and the prevalence of other ruminant dairy products in Mongol dietary traditions. In other documents, foreign visitors to the Mongol capital of Kharkorin noted the presence of a silver airag fountain, commissioned by one of the Khans, and meant to dispense fermented horse milk and wine to members of the Mongol elite [39]. Artistic and written depictions of this infamous fountain are common, through no physical remains of the fountain have been recovered.

During the Xiongnu period, there is additional historic evidence for ruminant and equine dairy consumption, although these texts were not written by Xiongnu scribes, but rather by members of Han Chinese groups who were frequently in contact with the Xiongnu. These texts (ca. 100 BCE - 200 CE) specifically mention the consumption of dairy products, but also the drinking of *lao*, which translates to "horse alcohol", at important Xiongnu social and political events [40]. Archaeologically, Xiongnu period ceramics indicative of cheese-making straining vessels suggest the manufacture of dairy products, and zooarchaeological evidence of pastoral kill-off patterns suggests dairy-focused subsistence practices were common in their multi-resource economies [33].

Without the guidance of historic documents for the time periods prior to the Xiongnu period in Mongolia, we have only archaeological evidence to consider. During the Bronze Age, there are few instances of occupation sites with recovered material objects in Mongolia [36,37]. Rather, the archaeological landscape is dominated by stone mounds, which often contain human burials [41]. Faunal remains of dairy animals are occasionally also recovered from satellite mounds placed around the primary human inhumations, yet these remains are often very fragmentary or charred, making it difficult to determine whether the animals were domesticated or wild [37,42]. While few occupation sites have been recovered and published to date, this trend is changing as more intensive survey techniques have been used to locate areas of prehistoric human domestic activity [37].

Prior to the Early Bronze Age in Mongolia very little is known about settlement structure or subsistence patterns largely due to the lack of the excavated sites prior to the Bronze Age. It has been suggested that early domesticated cattle *could* have been present in south-eastern Neolithic Mongolia [43], but these claims have not been substantiated with documented zooarchaeological methodologies and the faunal remains have been lost [44]. It is widely assumed that most Neolithic populations practiced hunting, gathering, and fishing, but this is difficult to substantiate due to the dearth of archaeological materials and skeletal evidence [43–45]).

1.3 *Grain cultivation in Central and Eastern Asia*

Mongolia's economy today is heavily focused on animal products, but crop cultivation, in the form of grains, potatoes, and other vegetables, provides an important part of modern Mongolian diets. Only a small percentage of the total landmass is arable (between 1 and 9.5% per aimag), and the arid climate as well as short growing seasons makes crop cultivation challenging [46]. Due to the small amount of land available for crop agriculture, many cereal grains and grain products are imported into Mongolia from nearby Central Asian countries of Russia and Kazakhstan [47].

Mongolia's historic imperial systems have long been viewed as populations of horseback raiders, dependent on dairy pastoralism, who primarily extorted outside resources through military intimidation and extortion from neighbouring populations. However, others have suggested these empires were far more intricate, with complex economic systems including grain agriculture, craft specialization, and extensive trade networks [33,48]. When these systems were first put into place is unknown, and an assumption of a specialized reliance on pastoral products has pervaded archaeological approaches to Mongolian societies and empires, often leading to them having a unique position in global comparisons of imperial economies. Cereal grain cultivation was undertaken during the Middle and Late Bronze Age in Central Asian regions surrounding Mongolia, but is not known to have been grown within its modern borders. Excavations from second and first millennium sites in southern Siberia, Kazakhstan, and the Xinjiang region of northwest China contain archaeobotanical evidence for millet (broomcorn and foxtail), as well as other grains (Figure 3) [49–53].

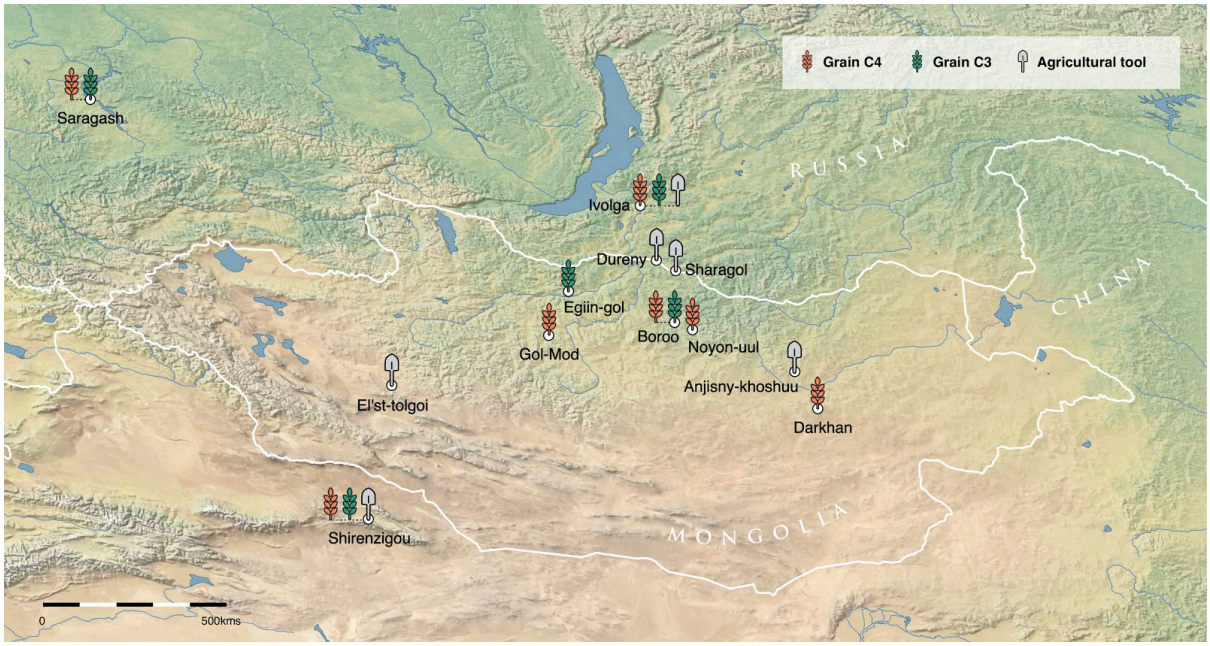

Figure 3. Locations of Iron Age sites with archaeological or botanical evidence for grain production and consumption (Wilkin et al., 2020 b; Manuscript C).

During Mongolia's imperial periods there is ample historic and archaeobotanical evidence for the presence of locally cultivated grains and internationally traded crops [13,34,54]. For example, unprocessed millet grains have been recovered from within the burials of Xiongnu rulers and other elites [55,56], suggesting that they were likely grown locally rather than traded over long distances [54]. Previously published stable isotope analysis and recovered archaeological material evidence, in the form of plowshares [57], suggest active, local cultivation and consumption which is further supported by historical recountings of administrative roles assigned to monitor millet production and distribution [40]. There remains a lack of direct archaeobotanical evidence for C_4 grain cultivation and consumption before the Iron Age, meaning that the time period when this grain was first included in eastern steppe diets remains a mystery. Through the application of stable isotope analysis to 155 individuals from prehistory to the Mongol period, we hope to add to the understanding of the range C_3 and C_4 grain consumption of each period, and identify differences between prehistoric and imperial subsistence economies. These data will add to the growing corpus of evidence detailing the importance of pastoral resource systems in early populations, as well as the complexities of preindustrial Mongolian empires.

1.4 *Methods used in this study*

1.4.1 *The history and methodologies used in ancient protein research*

The presence of proteins in archaeological materials has been studied since the 1950s, and the methods with which proteinaceous materials have been identified have evolved over the past 70 years. Initially, protein analysis was used to identify the presence of amino acids in fossils [58] and to determine the relative age of archaeological and paleontological materials containing amino acids, through a method known as amino acid racemization [59,60]. Other early protein studies delved into the likelihood of proteins remaining present in dinosaur bones [61], spurring the realization that while still affected by diagenesis, proteins could persist longer than DNA in the archaeological record. Immunoassays allowed for the identification of proteins by 1974, albeit only those that were specifically targeted for, and Edman sequencing, developed in the early 1980's, worked well with undamaged modern samples, but is not a reliable method for use with ancient degraded proteins [62].

The incorporation of mass spectrometry into protein studies has opened new avenues in the identification ancient peptide sequences, and has been heavily applied to zooarchaeological and palaeontological materials to investigate evolutionary and taxonomic questions, which is especially important when aDNA analysis is no longer possible [63–68]. One key development in the history of this field has been the use of Matrix Assisted Laser Desorption Ionization mass spectrometry (MALDI-MS) on collagen peptides extracted from palaeoanthropological and palaeofaunal skeletal remains, enabling the taxa-specific identification of bone collagen through a peptide mass fingerprinting approach [63,64,69]. The subsequent development and application of tandem mass spectrometry (MS/MS), and the development of 'shotgun proteomics' allowed researchers to identify the specific amino acid sequences of digested peptides, where an entire proteome could be recovered [70,71]. More recently, this method has been applied to archaeological materials beyond bone, such as dental calculus [72–74], ceramic residues and matrices [14,75], preserved organic materials [20,76], cultural heritage items [77], and mummified tissues [78].

Over the past 10 years, ancient protein analysis methods have become more widely applied, and recently researchers have explored some of the optimal strategies for identifying

endogenous proteins and generating replicable, shared data [79]. A number of methods have been developed enabling the extraction and identification of ancient proteins [74,80–83] (For a more detailed description protein extraction methods used in these studies see: Supplementary Information in Manuscript 1). Broadly, all these 'bottom-up' methods involve protein extraction, denaturation, digestion and purification before mass spectrometry analysis. Purified peptides are ionized and injected into an MS/MS via liquid chromatography (LC). During the MS/MS analysis, the mass over charge (m/z) of each peptide is measured; occurring rapidly as the sample's peptides are ionized and sent into the machine. The most frequently identified precursor ions (charged peptides) are then sent on for fragmentation and each of the resulting fragments is measured again by a mass analyser (Figure 4). Once the m/z of each of the precursor ions (MS1), and each of their daughter fragments (MS2) are known, the amino acid sequence of a peptide can be inferred by analysing mass shifts corresponding to amino acid losses in the daughter ions (Figure 5) [70]; For a more detailed description MS/MS methods and settings used in these studies see Supplementary Information in Manuscripts 1 and 2).

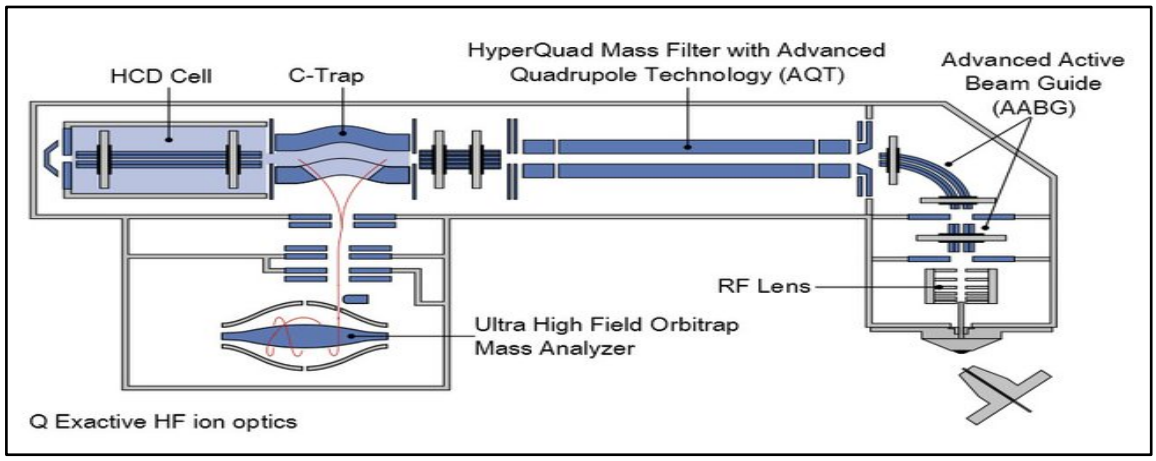

Figure 4: Diagram of a Thermo Scientific Q-Exactive HF tandem mass spectrometer (From Thermo Scientific at: https://www.thermofisher.com/order/catalog/product/IQLAAEGAAPFALGMBFZ)

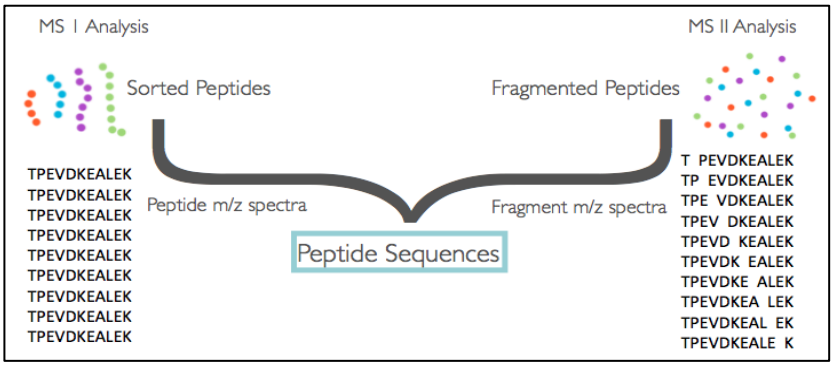

Figure 5. Illustration of how tandem mass spectrometry identifies peptides through two rounds of mass over charge measurements (m/z)

1.4.2 *Identifying milk proteins in ancient dental calculus*

During the past five years, ancient protein analysis using tandem mass spectrometry has been applied to human dental calculus in order to identify dietary [73,74,84,85] and human oral microbiome proteins [72,86]. Excitingly, this form of protein analysis has offered an indirect form of zooarchaeology by uncovering ancient human-animal interactions through the presence of animal dietary proteins and this has been especially important in cases where associated faunal remains are absent (Manuscript A; Manuscript B). As the studies included in this dissertation are specifically focused on dietary information from dental calculus, other methods focused on microbial or pathogenic protein identification will not be discussed here (See: [72,85–87]). Importantly, milk has a high protein content when compared to other beverages (between 1-14%), yet this varies widely by species (Table 6). It also has a distinctive protein contribution. The majority of proteins found in milk are caseins (alpha-S1-, alpha-S2-, beta-, and kappa-), followed by whey proteins (beta-lactoglobulin [BLG], alpha-lactalbumin, serum albumin, immunoglobulins) and others (lysozyme, lactoferrin, lactoperoxidase).

Studies into dietary proteins from ancient dental calculus have often identified dairy proteins, with examples of ruminant milk recovered from multiple archaeological contexts [70,81,82]. Ruminant milk protein sequences are largely conserved across the infraorder Pecora (even-toed hoofed mammals with ruminant digestion), with a few important differences between specific genera and species. While the peptides from largely conserved regions of a protein can determine that the identified milk protein was from an animal from the overall Pecora infraorder, for example, other peptides contain sequence differences that can identify the protein as deriving specifically from Ovis (sheep) or Capra (goat).

Interestingly, beta-lactoglobulin (BLG) is not produced by humans, and offers a way to specifically look at a protein only found in animal milks (Figure 7).

Species	Fat	Protein	Lactose	Ash	Total Solids
Ass (donkey)	1.3	6.9	4	1.3	25.2
Bison	1.7	4.8	5.7	0.96	13.2
Camel	4.9	3.7	5.1	0.7	14.4
Cow (Holstein)	3.5	3.1	4.9	0.7	12.2
Cow (Zebu)	4.9	3.9	5.1	0.8	14.7
Goat	3.5	3.1	4.6	0.79	12
Human	4.5	1.1	6.8	0.02	12.6
Horse	4.6	2.7	6.1	0.51	11
Reindeer	22.5	10.3	2.5	1.4	36.7
Sheep	5.3	5.5	4.6	0.9	16.3

Figure 6: Solid content variation between commonly milked animal species [30]

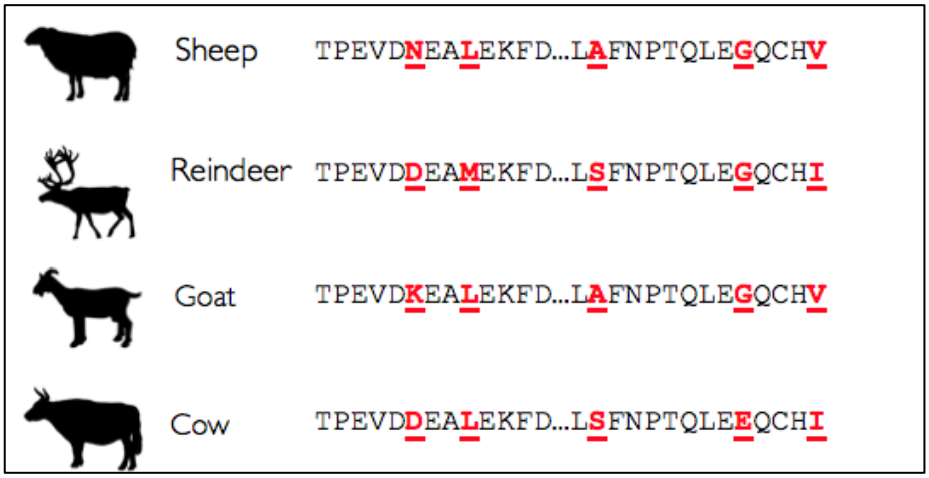

Figure 7. Differences in amino acid sequences of two commonly recovered peptides from beta-lactoglobulin, the most frequently recovered dairy protein. Each letter represents a specific amino acid, and those in red are those which indicate species specificity.

Due to the deep evolutionary divergence between ruminants and equines, there are proteins which are present in horse milk but not in the milk of other ruminants (Figure 8). For example, horses produce BLG II, which remains genetically present in ruminants, but is no longer expressed into proteins, and lysozyme C, a specific isozyme identifiable to horses [30,88]. Camels lack the ability to express proteins (BLG) that ruminants and horses produce, yet they synthesize others that have been specifically identified in camel milk (peptidoglycan recognition protein 1) [89]. Horse and camel milk contain high percentages of lactose and have low casein content in comparison to other dairy species, causing these milks to be difficult to process into other products such as cheeses or yogurts (Table 6). These same factors present both horse and camel milk as ideal materials for fermentation into alcoholic beverages. To date, in protein extraction from human dental calculus beta-lactoglobulin (BLG) is the most abundantly recovered milk protein, with alpha-S1-casein second, and an aggregate of other proteins following at far lower frequencies [73,74,84]. This could be due to the types of dairy preferred in the past (liquid whey rather than curd), but it seems more likely that this is due to the structural stability of BLG [90], allowing it to persist through time, or the ability of specific peptides to be recovered through current methods.

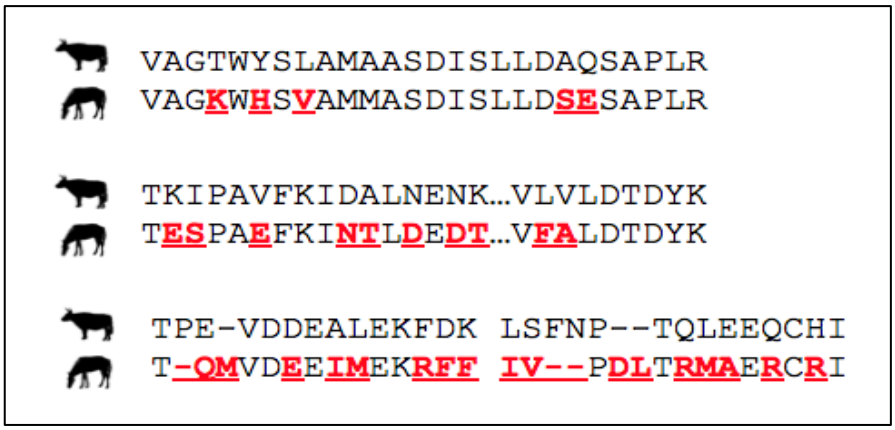

Figure 8. As in Figure 2, each letter represents an amino acid, and the red, underlined letters are those that differ between ruminants (Bos shown here) and equines. The first two lines show sequences that are conserved between all common ruminant dairy species, but do contain differences when comparing ruminant with horse peptides.

1.4.3 *History and application of stable carbon and nitrogen isotope analysis*

The application of stable isotope analysis to dietary studies extends back to the early 1960's, when the difference in $^{13}C/^{12}C$ ratio between C_3 and C_4 photosynthetic pathways was discovered. The fact that these values don't overlap indicates that this analysis can be used to

differentiate C_3 and C_4 plants from each other [91]. This idea was then applied to animals that consumed C_3 and C_4 plants, revealing that the $^{13}C/^{12}C$ ratio of the types of plants eaten would be measurable in the bones on the consumer [92]. Shortly after this discovery, it was demonstrated that $^{15}N/^{14}N$ stable isotope ratios of consumers vary depending on the trophic level of their food source(s) with a 3 ‰ addition per level, for example, an herbivore will have a $\delta^{15}N$ ratio that is 3 ‰ higher than that of the plants they consume [93]. While these methods were first applied to modern plants and animals, it can also be used on archaeological skeletal remains that contained adequate amounts of collagen.

$\delta^{13}C$ variability in terrestrial ecosystems is primarily driven by two dominant photosynthetic pathways, C_3 (wheat, barley, most wild plants) and C_4 (millet, maize, most of the remaining wild plants), which differ in their net discrimination against ^{13}C during photosynthesis [94]. These biological and ecological distinctions are passed into the tissues of consumers depending on the proportion of plants, and consumers of different plants that were consumed. Significantly, $\delta^{13}C$ analysis of human bone collagen primarily reflects the isotopic values of the protein input to the diet, with a minor contribution from lipids and carbohydrates [95]. This means that the $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen will be heavily influenced by protein-rich foods [95,96]. The rate of replacement of bone cells varies by skeletal element, and provide information over a specific time period, generally the last 15-20 years of the individual's life [97]. In contrast to bone collagen, $\delta^{13}C$ measurements of tooth enamel bioapatite reflect the carbon in the 'whole-diet' (proteins, carbohydrates, lipids, etc.) during the period of life of the enamel formation, and this varies depending on the species and which tooth is sampled [98].

In bone collagen, stable nitrogen isotope ratios ($\delta^{15}N$) provide additional dietary insights. The trophic effect is seemingly a result of the loss of ^{15}N-depleted products during excretion, though it should be noted that diet-tissue distinctions are highly variable between animals [99,100]. Freshwater fish typically have higher $\delta^{15}N$ values than terrestrial fauna due to the fact that aquatic systems often have longer food-chains, although $\delta^{13}C$ values of terrestrial and freshwater sources often overlap [96]. The $\delta^{15}N$ of plants, as well as their consumers, also has the potential to be influenced by environmental conditions, including aridity, salinity, and soil fertility [101–103] (for a more detailed background and methods used in this thesis, see the Supplementary Text 1 of Manuscript 3).

These methodologies have also been widely used in the Holocene to identify dietary patterns and economies. For example, carbon values have been used to identify early North American maize consumers (C_4 pathway), distinguishing them from consumers of a hunter-gatherer diet (primarily C3 pathways) [104–106]. Differences in the carbon values of certain members of populations has also been used to respond to questions of social inequities visible through differences in dietary intake, for example, those in elite burials likely subsisted on more nutritious meats over the less nutrient-dense maize that was widely available [107]. Studies, such as that from the midwestern individuals from the modern day United States, detailed a continued reliance on maize products, but could also indicate that diets also contained fish due to the change in trophic level indicated by the $\delta^{15}N$ ratios [108].

Recently, stable isotope analysis of archaeological human and associated faunal remains has emerged as an increasingly powerful methodology for tracking paleodietary and subsistence changes in Central and East Asia. Through large-scale studies of individuals over time, we can see broad changes in dietary intake, especially changes in C_3 and C_4 grain consumption. Studies using archaeobotanical evidence combined with stable carbon isotope ratios of western, northern, and central Eurasian Steppe zones human [109–111] and animal herd populations [112] detail the widespread use of wheat, barley, and millet across most of the steppe, and a lack of evidence in Mongolia until the Iron Age [111,113].

2. Aims of the thesis

The combination of proteomic and stable isotope biomolecular analyses incorporated into existing archaeological and historic evidence offers insights into individual and population-level trends in dietary intake and economic diversity. Here, we illuminate the evolution of subsistence strategies through environmental, social, and political transformations over 5000 years of Mongolia's past through the following three aimed studies.

We aim to determine whether Late Bronze Age (*c.* 1500 - 800 B.C.E.) individuals in northern Mongolia were consuming dairy products, and if the dairy taxa identified in this approach align with associated zooarchaeological evidence. We expect that dairy pastoralism was common by the Late Bronze Age, and would consist of primarily sheep and goat dairy resources. We also include an ancient DNA analysis to determine whether northern Mongolian dairying populations had local or western steppe genetic ancestry.

To determine the development and diversity of Mongolia's dairy traditions, through the identification of milk proteins recovered from ancient human dental calculus. Through the extraction and analysis of species-specific peptides we demonstrate ancient and historic human-animal interactions. These data will be coordinated with established archaeological and historic evidence to contextualize early dairying practices. Based on current archaeological knowledge, we hypothesize ruminant dairy to be present in the dental calculus beginning in the Late Bronze Age, and that horse milk proteins will first be identified during the Xiongnu period (ca. 200 BCE – 200 CE).

We will assess the importance of C_4 grain consumption and cultivation through stable carbon and nitrogen isotope analysis from bone collagen, and carbon isotope analysis from dental enamel bioapatite. Resulting data from the Xiongnu, Mongol (*c.* 1200 -1400 CE), and pre-imperial populations will be combined with archaeological, botanical, and historical evidence to explore and compare the dietary complexities of each period. We hypothesize that millet, or other C_4 grain consumption, would become visible through stable carbon isotope analysis and archaeobotanical evidence recovered from Xiongnu period.

3. Manuscript A

"Bronze Age population dynamics and the rise of dairy pastoralism on the eastern Eurasian steppe"

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This study focused on the proteomic analysis of dental calculus in combination with ancient DNA analysis. This project aimed to test for the presence of dairy consumption in north central Mongolia during the Late Bronze Age while also conducting genome analysis to determine whether the population was purely local or shared ancestry with western steppe populations. The following specific analyses were performed:

- Protein extraction and analysis of nine distinct individuals from sites across the Khovsgol aimag of northern Mongolia spanning between 1380 - 975 BCE.
- Ancient DNA analysis of 20 individuals from the same aimag and time period
- Comparison of protein data to faunal remains common in Mongolia's Late Bronze Age
- Comparison of genomic data to known western steppe and local ancient northern Eurasian populations to determine ancestry

Author contributions: Choongwon Jeong, Frank J. Rühli, Bruno Frohlich, Jessica Hendy and Christina Warinner designed the research plan. Choongwon Jeong, Shevan Wilkin, Tsend Amgalantugs, Abigail S. Bouwman, Richard W. Hagan, Sabri Bromage, Soninkhishig Tsolomon, Christian Trachsel, Judith Littleton, John Krigbaum, Marta Burri, Ganmaa Davaasambuu, Fransizka Irmer, Erdene Myagmar, Bruno Frohlich, Jessica Hendy, and Christina Warinner performed research; Richard W. Hagan, Christian Trachsel, Jonas Grossmann, and Ashely Scott contributed new reagents/analytic tools; Nicole Boivin, Martine Robbeets, and Johannes Krause contributed personnel and material resources; Choongwon

Jeong, Shevan Wilkin, William T.T. Taylor, Richard W. Hagan, Sabri Bromage, Soninkhisig Tsolomon, Christian Trachsel, Jonas Grossmann, Cheryl A. Makarewicz, John Krigbaum, Ashley Scott, Ganmaa Davaasambuu, Joshua Wright, Nicole Boivin, Martine Robbeets, Johannes Krause, Bruno Frohlich, Jessica Hendy, and Christina Warinner analyzed data; and Choongwon Jeong, Shevan Wilkin, Jessica Hendy and Christina Warinner wrote the paper. In total, Shevan Wilkin contributed to 50% of the project.

Bronze Age population dynamics and the rise of dairy pastoralism on the eastern Eurasian steppe

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Recent paleogenomic studies have shown that migrations of Western steppe herders (WSH) beginning in the Eneolithic (ca. 3300–2700 BCE) profoundly transformed the genes and cultures of Europe and central Asia. Compared with Europe, however, the eastern extent of this WSH expansion is not well defined. Here we present genomic and proteomic data from 22 directly dated Late Bronze Age burials putatively associated with early pastoralism in northern Mongolia (ca. 1380–975 BCE). Genome-wide analysis reveals that they are largely descended from a population represented by Early Bronze Age hunter-gatherers in the Baikal region, with only a limited contribution (∼7%) of WSH ancestry. At the same time, however, mass spectrometry analysis of dental calculus provides direct protein evidence of bovine, sheep, and goat milk consumption in seven of nine individuals. No individuals showed molecular evidence of lactase persistence, and only one individual exhibited evidence of >10% WSH ancestry, despite the presence of WSH populations in the nearby Altai-Sayan region for more than a millennium. Unlike the spread of Neolithic farming in Europe and the expansion of Bronze Age pastoralism on the Western steppe, our results indicate that ruminant dairy pastoralism was adopted on the Eastern steppe by local hunter-gatherers through a process of cultural transmission and minimal genetic exchange with outside groups.

paleogenomics | LC-MS/MS | dental calculus | β-lactoglobulin | α-S1-casein

Archaeogenetic studies provide evidence that the Eurasian Eneolithic–Bronze Age transition was associated with major genetic turnovers by migrations of peoples from the Pontic-Caspian steppe both in Europe and in central Asia (1–5). The migration of these Western steppe herders (WSH), with the Yamnaya horizon (ca. 3300–2700 BCE) as their earliest representative, contributed not only to the European Corded Ware culture (ca. 2500–2200 BCE) but also to steppe cultures located between the Caspian Sea and the Altai-Sayan mountain region, such as the Afanasievo (ca. 3300–2500 BCE) and later Sintashta (2100–1800 BCE) and Andronovo (1800–1300 BCE) cultures. Although burials typologically linked to the Afanasievo culture have been occasionally reported in Mongolia (6), the genetic profile of Eastern steppe populations, as well as the timing and nature of WSH population expansion and the rise of dairy pastoralism in Mongolia, remain unclear.

The remarkable demographic success of WSH populations has been linked to mobile pastoralism with dairying (7), a system that efficiently converts cellulose-rich wild grasses into protein- and fat-rich dairy products. Dairy foods provide a rich source of nutrients and fresh water, and function as an adaptive subsistence strategy in cold, dry steppe environments where most crop cultivation is highly challenging. Dairy pastoralism became widely practiced in the eastern Eurasian steppe, the homeland from which subsequent historical nomadic dairying empires, such as the Xiongnu (ca. 200 BCE to 100 CE) and the Mongols (ca. 1200–1400 CE) expanded; however, it is not fully understood when, where, and how this subsistence strategy developed. At

Significance

Since the Bronze Age, pastoralism has been a dominant subsistence mode on the Western steppe, but the origins of this tradition on the Eastern steppe are poorly understood. Here we investigate a putative early pastoralist population in northern Mongolia and find that dairy production was established on the Eastern steppe by 1300 BCE. Milk proteins preserved in dental calculus indicate an early focus on Western domesticated ruminants rather than local species, but genetic ancestry analysis indicates minimal admixture with Western steppe herders, suggesting that dairy pastoralism was introduced through adoption by local hunter-gatherers rather than population replacement.

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Data deposition: The sequences reported in this paper have been deposited in the NCBI Sequence Read Archive (SRA) (bioproject accession no. PRJNA429081). The protein spectra have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (accession no. PXD008217).

See Commentary on page 12083.

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Botai, in central Kazakhstan, evidence for Eneolithic dairying has been reported through the presence of ruminant and equine dairy lipids in ceramic residues as early as 3500 BCE (8, 9). In the Altai and Tarim basin, where WSH populations have left strong genetic footprints (1, 3, 10, 11), archaeological evidence supports the presence of dairy products by the early second Millennium BCE and later (8, 12, 13). In the Eastern steppe, however, no direct observations of dairy consumption have been made for a comparable time period, despite the fact that skeletal remains of domestic livestock (such as sheep, goats, cattle, and horses) have been found at Mongolian ritual sites and in midden contexts as early as the 14th century BCE (14–17). In the absence of direct evidence for Bronze Age milk production or consumption on the Eastern steppe, it remains unclear whether these animals are merely ritual in nature or signify a major shift in dietary ecology toward dairy pastoralism, and whether their appearance is connected to possible WSH migrations onto the Eastern steppe.

To understand the population history and context of dairy pastoralism in the eastern Eurasian steppe, we applied genomic and proteomic analyses to individuals buried in Late Bronze Age (LBA) burial mounds associated with the Deer Stone-Khirigsuur Complex (DSKC) in northern Mongolia (*SI Appendix*, Figs. S1–S3 and Table S1). To date, DSKC sites contain the clearest and most direct evidence for animal pastoralism in the Eastern steppe before ca. 1200 BCE (18). Focusing on six distinct burial clusters in Arbulag soum, Khövsgöl aimag, Mongolia (Fig. 1 and *SI Appendix*, Figs. S1–S3), we produced genome-wide sequencing data targeting ∼1.2M single nucleotide polymorphisms (SNPs) for 22 DSKC-associated individuals directly dated to ca. 1380–975 calibrated BCE (*SI Appendix*, Fig. S4 and Table S2), as well as sequenced whole genomes for two individuals (>3× coverage). Nine of the individuals in this group yielded sufficient dental calculus for proteomic analysis, and we tested these deposits for the presence of milk proteins using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Overall, our results find that DSKC subsistence strategy included dairying of Western domesticated ruminants, but that there was minimal gene flow between analyzed DSKC populations and WSH groups during the LBA. Thus, in contrast to patterns observed in western Europe where, for example, the arrival of WSH is associated with population replacement and continental-level genetic turnover (5), contact between WSH and Eastern steppe populations is characterized by transcultural transmission of dairy pastoralism in the near absence of demic diffusion.

Results

Ancient DNA Sequencing and Quality Assessment. We built and sequenced uracil-DNA-glycosylase–half (19), double-indexed Illumina libraries for genomic DNA extracted from teeth or femora from DSKC-associated burials in Khövsgöl, Mongolia. Twenty of 22 libraries exhibited good human DNA preservation, with a mean host endogenous content of 14.9% (range 0.2–70.0%); two libraries yielded very little human DNA (<0.05%) and were excluded from further analysis (*SI Appendix*, Table S2). Libraries were then enriched for 1.2 million variable sites in the human genome (1240K) using in-solution hybridization (2, 3). All individuals (12 males, 8 females) showed characteristic patterns of chemical modifications typical of ancient DNA (*SI Appendix*, Fig. S5), and 18 individuals yielded both low estimates of modern DNA contamination (≤1% mitochondrial and nuclear contamination) and sufficient genome coverage for subsequent analysis (0.11× to 4.87× mean coverage for target sites) (*SI Appendix*, Table S3). No close relative pairs were identified among the ancient individuals (*SI Appendix*, Fig. S6). Two individuals with high endogenous content on screening (ARS008, 70.0%; ARS026, 47.6%) were deeply sequenced to obtain whole genomes (∼3.3× coverage) (*SI Appendix*, Table S3). We intersected our ancient data with a published world-wide set of ancient and contemporary individuals (Dataset S1) whose genotypes are determined for 593,124 autosomal SNPs on the Affymetrix HumanOrigins 1 array (20).

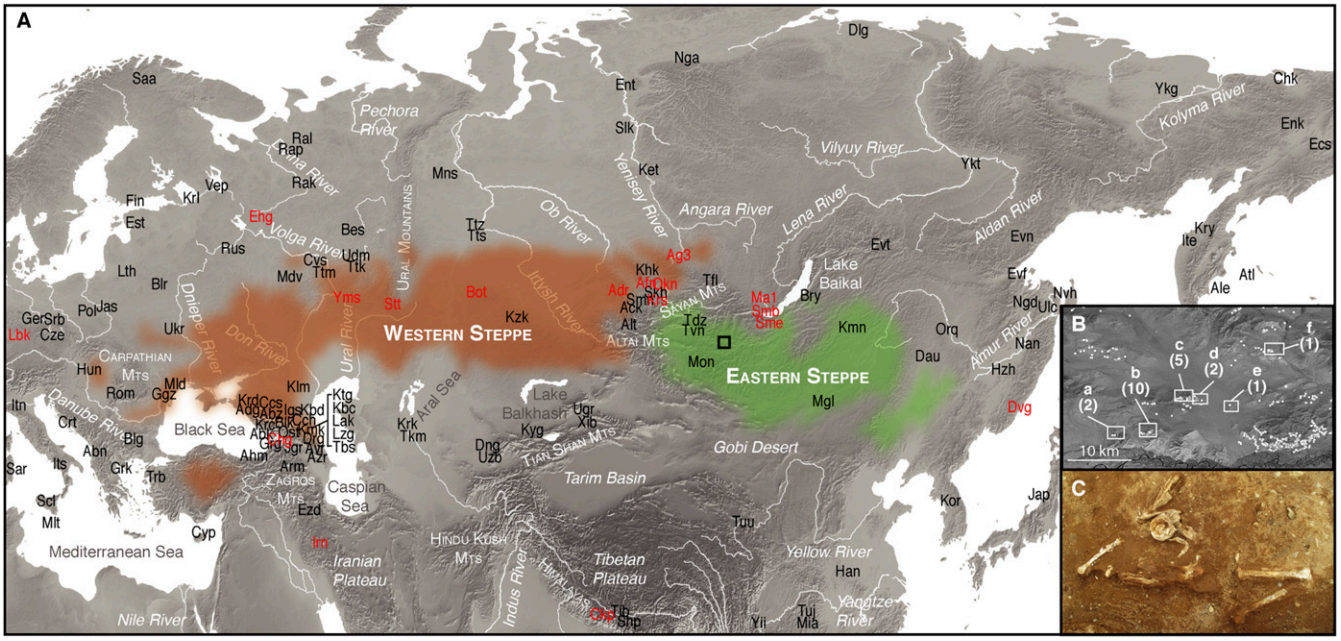

Fig. 1. Map of the Eurasian steppes. (*A*) Distribution of the Western (brown) and Eastern (green) steppes and the locations of ancient (red) and modern (black) populations discussed in the text. Population codes are provided in the Dataset S1. A box indicates the location of the LBA burial mounds surveyed in the Arbulag soum of Khövsgöl aimag. (*B*) Enhanced view of LBA burial mounds (white circles) and burial clusters selected for excavation (boxes *a*–*f*) with the number of analyzed individuals in parentheses (*SI Appendix*, Table S1). (*C*) Photograph of burial 2009-52 containing the remains of ARS026, a genetic outlier with Western steppe ancestry.

Characterization of the Genetic Profile of the Khövsgöl Gene Pool. To characterize the genetic profile of DSKC-associated LBA Khövsgöl individuals (Khövsgöls), we performed principal component analysis (PCA) of Eurasian populations (*SI Appendix*, Fig. S7). PC1 separates eastern and western Eurasian populations, with central and north Eurasian populations falling in an intermediate position (*SI Appendix*, Fig. S7). PC2 separates eastern Eurasian populations along a north–south cline, with northern Siberian Nganasans and the Ami and Atayal from Taiwan forming the northern and southern end points, respectively. Most LBA Khövsgöls are projected on top of modern Tuvinians or Altaians, who reside in neighboring regions. In comparison with other ancient individuals, they are also close to but slightly displaced from temporally earlier Neolithic and Early Bronze Age (EBA) populations from the Shamanka II cemetry (Shamanka_EN and Shamanka_EBA, respectively) from the Lake Baikal region (*SI Appendix*, Fig. S7) (4, 21). However, when Native Americans are added to PC calculation, we observe that LBA Khövsgöls are displaced from modern neighbors toward Native Americans along PC2, occupying a space not overlapping with any contemporary population (Fig. 2*A* and *SI Appendix*, Fig. S8). Such an upward shift on PC2 is also observed in the ancient Baikal populations from the Neolithic to EBA and in the Bronze Age individuals from the Altai associated with Okunevo and Karasuk cultures (1). These observations are consistent with LBA Khövsgöls and other ancient Siberians sharing more ancestry with Native American-related gene pools than modern populations in the region do.

Notably, two individuals fall on the PC space markedly separated from the others: ARS017 is placed close to ancient and modern northeast Asians, such as early Neolithic individuals from the Devil's Gate archaeological site (22) and present-day Nivhs from the Russian far east, while ARS026 falls midway between the main cluster and western Eurasians (Fig. 2*A*). Genetic clustering with ADMIXTURE (23) further supports these patterns (Fig. 2*B* and *SI Appendix*, Fig. S9). We quantified the genetic heterogeneity between Khövsgöl individuals by calculating f_4 symmetry statistics (24) in the form of f_4(chimpanzee, outgroup; Khövsgöl$_1$, Khövsgöl$_2$) for all pairs against 18 outgroups representative of world-wide ancestries (*SI Appendix*, Fig. S10). As expected, the two outliers did not form a clade with the rest of individuals and therefore we treated each individual separately in subsequent analyses. For the remaining 16 individuals, 14 were merged into a single main cluster based on their minimal genetic heterogeneity. The other two individuals (ARS009 and ARS015) were excluded from this cluster because they broke symmetry with four and two individuals (maximum

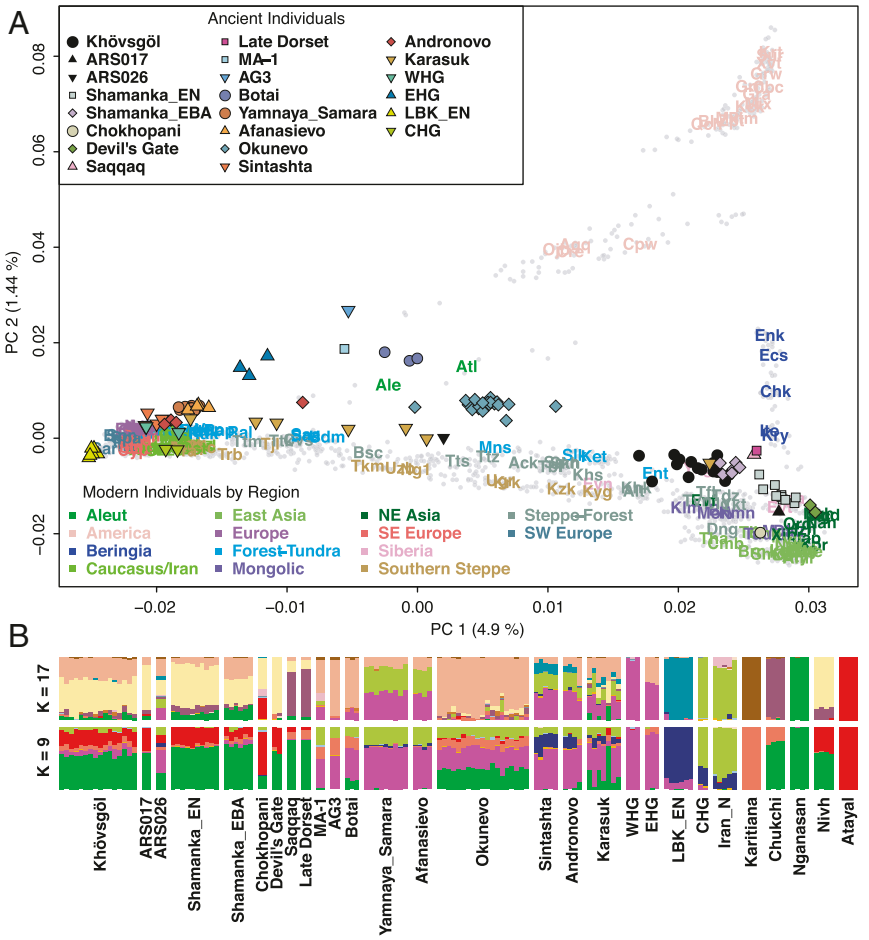

Fig. 2. The genetic profile of LBA Khövsgöl individuals summarized by PCA and ADMIXTURE. (*A*) Khövsgöl (Kvs, ARS017, and ARS026) and other ancient individuals (colored symbols) are projected onto the top PCs of modern Eurasian and Native American individuals. Contemporary individuals are marked by gray circles. Mean coordinates for each of the contemporary populations are marked by three-letter codes and by colors assigned to the associated geographic regions. Population codes are provided in Dataset S1 and *SI Appendix*, Fig. S8. (*B*) ADMIXTURE results for Khövsgöl and other ancient individuals with *K* values 9 and 17. In *K* = 17, the Khövsgöls main cluster is mainly modeled as a mixture of components most enriched in modern northeast Asians (e.g., Nivh) and ancient Siberians (e.g., AG3, Botai, and Okunevo).

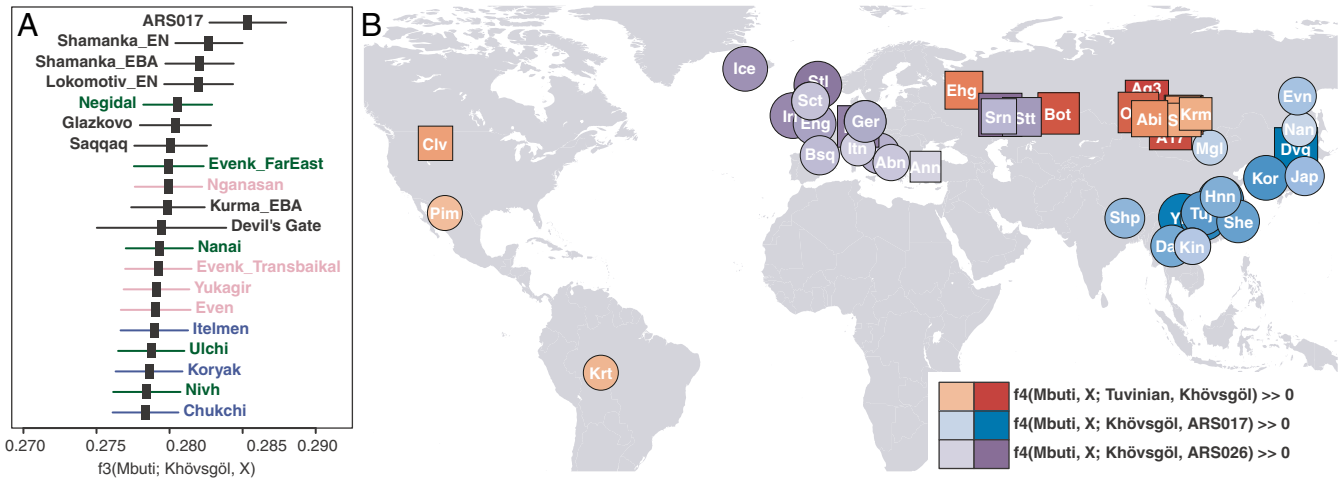

Fig. 3. The genetic affinity of the Khövsgöl clusters measured by outgroup-f_3 and -f_4 statistics. (*A*) The top 20 populations sharing the highest amount of genetic drift with the Khövsgöl main cluster measured by f_3(Mbuti; Khövsgöl, X). (*B*) The top 15 populations with the most extra affinity with each of the three Khövsgöl clusters in contrast to Tuvinian (for the main cluster) or to the main cluster (for the two outliers), measured by f_4(Mbuti, X; Tuvinian/Khövsgöl, Khövsgöl/ARS017/ARS026). Ancient and contemporary groups are marked by squares and circles, respectively. Darker shades represent a larger f_4 statistic. Population codes are provided in Dataset S1; see also *SI Appendix*, Figs. S11–S14 for further details.

$|Z|$ = 3.9 and 4.7 SE), respectively, and were also slightly displaced from the others in our PCA (Fig. 2*A*).

Next, we quantified the genetic affinity between our Khövsgöl clusters and world-wide populations by calculating outgroup-f_3 statistics with Central African Mbuti as an outgroup (25). For the main cluster, top signals were observed with earlier ancient populations from the Baikal region, such as the early Neolithic and EBA individuals from the Shamanka II cemetry (4), followed by present-day Siberian and northeast Asian populations, such as Negidals from the Amur River basin and Nganasans from the Taimyr peninsula (Fig. 3*A* and *SI Appendix*, Fig. S11 *A* and *B*). As expected based on their nonoverlapping positions on PCA, however, Khövsgöls do not form a cluster with these high-affinity groups, as shown by f_4 symmetry tests in the form of f_4(Mbuti, X; Siberian, Khövsgöls). Interestingly, Upper Paleolithic Siberians from nearby Afontova Gora and Mal'ta archaeological sites (AG3 and MA-1, respectively) (25, 26) have the highest extra affinity with the main cluster compared with other groups, including the eastern outlier ARS017, the early Neolithic Shamanka_EN, and present-day Nganasans and Tuvinians (Z > 6.7 SE for AG3) (red shades in Fig. 3*B* and *SI Appendix*, Fig. S11 *C* and *D*). This extra affinity with so-called "Ancient North Eurasian" (ANE) ancestry (27) may explain their attraction toward Native Americans in PCA, because Native Americans are known to have high proportion of ANE ancestry (20, 25). Main-cluster Khövsgöl individuals mostly belong to Siberian mitochondrial (A, B, C, D, and G) and Y (all Q1a but one N1c1a) haplogroups (*SI Appendix*, Table S4).

Source of ANE Ancestry in the LBA Khövsgöl Population. Previous studies show a close genetic relationship between WSH populations and ANE ancestry, as Yamnaya and Afanasievo are modeled as a roughly equal mixture of early Holocene Iranian/Caucasus ancestry (IRC) and Mesolithic Eastern European hunter-gatherers, the latter of which derive a large fraction of their ancestry from ANE (20, 28). It is therefore important to pinpoint the source of ANE-related ancestry in the Khövsgöl gene pool: that is, whether it derives from a pre-Bronze Age ANE population (such as the one represented by AG3) or from a Bronze Age WSH population that has both ANE and IRC ancestry. To test these competing hypotheses, we systematically compared various admixture models of the main cluster using the qpAdm program (20). Ancient Baikal populations were chosen as a proxy based on both their spatiotemporal and genetic similarities with the Khövsgöl main cluster (Figs. 2 and 3). When the early Neolithic Shamanka_EN is used as a proxy, we find that Baikal+ANE provides a better fit to the main cluster than Baikal+WSH, although no two-way admixture model provides a sufficient fit ($P \geq 0.05$) (*SI Appendix*, Table S5). Adding a WSH population as the third source results in a sufficient three-way mixture model of Baikal+ANE+WSH with a small WSH contribution to the main cluster (e.g., $P = 0.180$ for Shamanka_EN+AG3+Sintashta with 3.7 ± 2.0% contribution from Sintashta) (Fig. 4 and *SI Appendix*, Table S6).

Using the temporally intermediate EBA population Shamanka_EBA, we can narrow down the time for the introduction

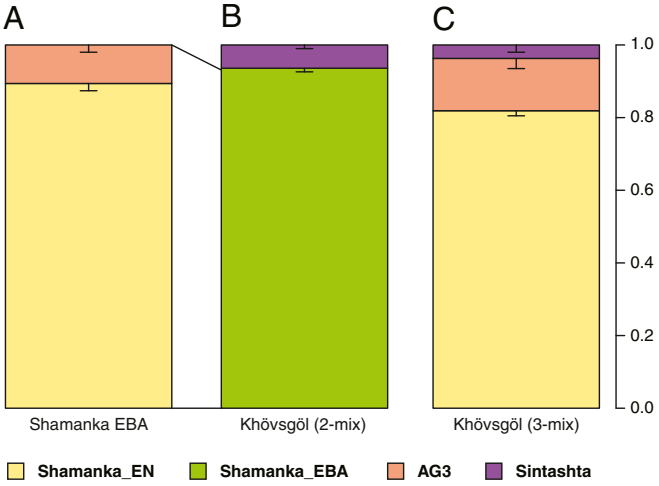

Fig. 4. Admixture modeling of Altai populations and the Khövsgöl main cluster using qpAdm. For the archaeological populations, (*A*) Shamanka_EBA and (*B* and *C*) Khövsgöl, each colored block represents the proportion of ancestry derived from a corresponding ancestry source in the legend. Error bars show 1 SE. (*A*) Shamanka_EBA is modeled as a mixture of Shamanka_EN and AG3. The Khövsgöl main cluster is modeled as (*B*) a two-way admixture of Shamanka_EBA+Sintashta and (*C*) a three-way admixture Shamanka_EN+AG3+Sintashta. Details of the admixture models are provided in *SI Appendix*, Tables S5 and S6.

of WSH ancestry into the main cluster. Shamanka_EBA is modeled well as a two-way mixture of Shamanka_EN and ANE (P = 0.158 for Shamanka_EN+AG3) (Fig. 4) but not as a mixture of Shamanka_EN and WSH ($P \leq 2.91 \times 10^{-4}$) (*SI Appendix*, Table S5), suggesting no detectable WSH contribution through the early Bronze Age. Similar results are obtained for other Late Neolithic and EBA populations from the Baikal region (*SI Appendix*, Table S5). In contrast, the Khövsgöl main cluster is modeled well by Shamanka_EBA+WSH but not by Shamanka_EBA+ANE ($P \geq$ 0.073 and $P \leq 0.038$, respectively) (*SI Appendix*, Table S5). A three-way model of Shamanka_EBA+ANE+WSH confirms this by providing the ANE contribution around zero (*SI Appendix*, Table S6). The amount of WSH contribution remains small (e.g., 6.4 ± 1.0% from Sintashta) (Fig. 4 and *SI Appendix*, Table S5). Assuming that the early Neolithic populations of the Khövsgöl region resembled those of the nearby Baikal region, we conclude that the Khövsgöl main cluster obtained ∼11% of their ancestry from an ANE source during the Neolithic period and a much smaller contribution of WSH ancestry (4–7%) beginning in the early Bronze Age.

Admixture Testing of Genetic Outliers. Using the same approach, we obtained reasonable admixture models for the two outliers, ARS017 and ARS026. The eastern outlier ARS017, a female, shows an extra affinity with early Neolithic individuals from the Russian far east (Devil's Gate) (22) and in general with contemporary East Asians (e.g., Han Chinese) compared with the Khövsgöl main cluster (Fig. 3*B* and *SI Appendix*, Fig. S12). ARS017 is also similar to Shamanka_EN in showing no significant difference in qpAdm (*SI Appendix*, Fig. S12 and Table S7). Using contemporary East Asian proxies, ARS017 is modeled as a mixture of predominantly Ulchi and a minor component (6.1–9.4%) that fits most ancient western Eurasian groups (P = 0.064–0.863) (*SI Appendix*, Table S7). This minor Western component may result from ANE ancestry; however, given the minimal western Eurasian contribution, we do not have sufficient power to accurately characterize this individual's western Eurasian ancestry.

The Western outlier ARS026, a male dating to the end of the radiocarbon series, has the highest outgroup-f_3 with the main LBA Khövsgöl cluster, with extra affinity toward Middle Bronze Age (MBA) individuals from the Sintashta culture (Fig. 3*B* and *SI Appendix*, Fig. S13) (1). DNA recovered from this individual exhibited expected aDNA damage patterns (*SI Appendix*, Fig. S5) but was otherwise excellently preserved with >47% endogenous content and very low estimated contamination (1% mitochondrial; 0.01% nuclear). ARS026 is well modeled as a two-way mixture of Shamanka_EBA and Sintashta (P = 0.307; 48.6 ± 2.0% from Sintashta) (*SI Appendix*, Table S7). Similar to ARS026, contemporaneous LBA Karasuk individuals from the Altai (1400–900 BCE) (1, 29) also exhibit a strong extra genetic affinity with individuals associated with the earlier Sintashta and Andronovo cultures (*SI Appendix*, Fig. S14). Although two-way admixture models do not fit ($P \leq 0.045$) (*SI Appendix*, Table S8), the Karasuk can be modeled as a three-way mixture of Shamanka_EBA/Khövsgöl and AG3 and Sintashta, suggesting an eastern Eurasian source with slightly higher ANE ancestry than those used in our modeling ($P \geq 0.186$) (*SI Appendix*, Table S8). Like ARS026, admixture coefficients for the Karasuk suggest that MBA/LBA groups like the Sintashta or Srubnaya are a more likely source of their WSH ancestry than the EBA groups, like the Yamnaya or Afanasievo. Notably, Karasuk individuals are extremely heterogeneous in their genetic composition, with the genetically easternmost Eurasian individual nearly overlapping with the EBA Baikal groups (Fig. 2*A* and *SI Appendix*, Figs. S7 and S8). Earlier groups, such as the Afanasievo, Sintashta, and Andronovo, are mostly derived from WSH ancestries, and this may suggest that admixture in the Altai-Sayan region only began during the LBA following a long separation since the Eneolithic. Although ARS026 exhibits substantial WSH ancestry, strontium isotopic values obtained from his M3 enamel resemble local fauna and fall within the range of the main Khövsgöl cluster (*SI Appendix*, Fig. S15 and Table S9); however, because the enamel this individual also exhibited elevated manganese levels, postmortem trace element alteration from soil could not be excluded.

Dairy Subsistence and Lactase Persistence. Contemporary Mongolia has a dairy- and meat-based subsistence economy, and to more precisely understand the role of dairy products in the diets of present-day mobile pastoralists in Khövsgöl aimag, we conducted a detailed nutritional investigation of summer and winter diets. We find that dairy-based foods contribute a mean of 35% total dietary energy, 36–40% total carbohydrate, 24–31% total protein, and 39–40% total fat to rural summer diets in Khövsgöl aimag, with liquid milk and dairy product consumption of 216–283 and 172–198 g/d, respectively (*SI Appendix*, Table S10 and Dataset S2).

Despite the importance of dairying today, its origins in Mongolia are poorly understood. Given the limited WSH ancestry of the main Khövsgöl cluster, we sought to determine if dairy pastoralism was practiced by this putatively pastoralist LBA population by testing for the presence of milk proteins (30) in the dental calculus of these individuals. We extracted proteins from 12 dental calculus samples representing 9 individuals (*SI Appendix*, Table S11) and analyzed tryptic peptides using LC-MS/MS (31). Observed modifications included deamidation (N, Q) and oxidation (P, M) (*SI Appendix*, Table S12). All protein identifications were supported by a minimum of two peptides across the dataset, and only peptides with an E value ≤ 0.001 were assigned; the estimated peptide false-discovery rate (FDR) across the full dataset was 1.0%, and protein FDR was 4.6%. Milk proteins were detected in seven of the nine individuals analyzed (*SI Appendix*, Table S13 and Dataset S3), confirming that dairy foods were consumed as early as 1456 BCE (1606–1298 BCE, 95% probability of the earliest directly dated individual) (*SI Appendix*, Fig. S4 and Table S2). Specifically, we detected the milk whey protein β-lactoglobulin (Fig. 5 *A* and *B*) and the curd protein α-S1-casein, with peptides matching specifically to sheep (*Ovis*), goat (*Capra*), Caprinae, Bovinae, and a subset of Bovidae (*Ovis* or Bovinae) (Fig. 5*C*, *SI Appendix*, Table S13, and Dataset S3). These peptides exhibited asparagine and glutamine deamidation, as expected for ancient proteins (32), and the frequency and distribution of recovered β-lactoglobulin (Fig. 5*B*) and α-S1-casein peptides closely matched that empirically observed for modern bovine milk (33), thereby providing additional protein identification support through appropriate proteotypic behavior.

Given the evidence for dairy consumption by the LBA Khövsgöl population, we sought to determine if the dairy-adaptive -13910*T (rs4988235) lactase persistence (LP) allele found today in Western steppe (34) and European (35) populations was present among LBA Khövsgöls dairy herders, and we examined this position in our SNP-enriched dataset. The -13910*T LP allele was not found in the LBA Khövsgöls (*SI Appendix*, Fig. S17 and Table S14), and additionally all observed flanking sequences in the lactase transcriptional enhancer region contained only ancestral alleles.

Discussion

In this study, we find a clear genetic separation between WSH populations and LBA Mongolians more than a millennium after the arrival of WSH at the furthest edges of the Western steppe and the earliest appearance of the WSH Afanasievo cultural elements east of the Altai-Sayan mountain range. This genetic separation between Western and Eastern steppe populations appears to be maintained with very limited gene flow until the

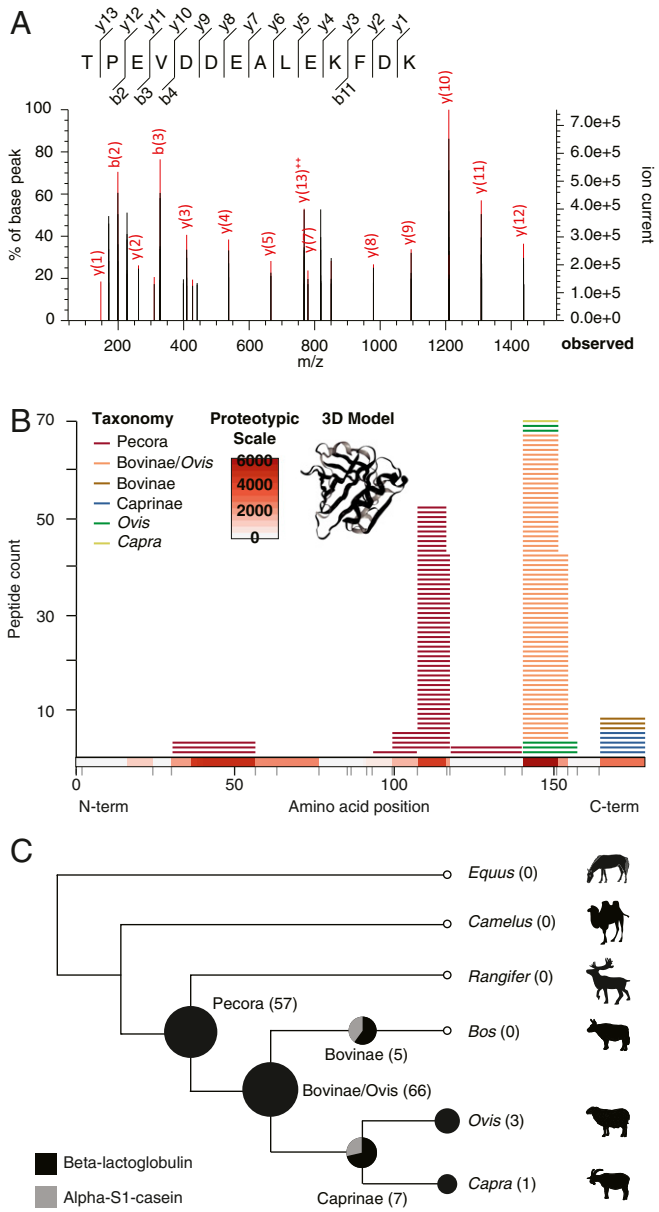

Fig. 5. Presence of ruminant β-lactoglobulin and α-S1-casein milk protein in LBA Khövsgöl dental calculus. (*A*) B- and Y-ion series for one of the most frequently observed β-lactoglobulin peptides, TPEVD(D/N/K)EALEKFDK, which contains a genus-specific polymorphic residue: D, *Bos*; N, *Ovis*; K, *Capra*. See *SI Appendix*, Fig. S16 for peptide and fragment ion error distribution graphs. (*B*) Alignment of observed peptides to the 178 amino acid β-lactoglobulin protein, with peptide taxonomic source indicated by color. Trypsin cut sites are indicated by gray ticks. The position and empirically determined observation frequency of BLG peptides for bovine milk are shown as a heatmap scaled from least observed peptides (light gray) to most frequently observed peptides (dark red), as reported in the Bovine PeptideAtlas (34). *Inset* displays a 3D model of the β-lactoglobulin protein with observed peptide positions highlighted in black. (*C*) Taxonomically assigned β-lactoglobulin (black) and α-S1-casein (gray) peptides presented as scaled pie charts on a cladogram of Mongolian dairy domesticates. Bracketed numbers represent the number of peptides assigned to each node. Ruminant milk proteins were well supported, but no cervid, camelid, or equid milk proteins were identified.

end of the LBA, when admixed populations, such as the Karasuk (1200–800 BCE), first appear in the Altai (1) and we observe the first individual with substantial WSH ancestry in the Khövsgöl population, ARS026, directly dated to 1130–900 BCE. Consistent with these observations, we find that the WSH ancestry introduced during these admixture events is more consistent with MBA and LBA steppe populations, such as the Sintashta (2100–1800 BCE), than with earlier EBA populations, such as the Afanasievo (3300–2500 BCE), who do not seem to have genetically contributed to subsequent populations.

Despite the limited gene flow between the Western and Eastern steppes, dairy pastoralism was nevertheless adopted by local non-WSH populations on the Eastern steppe and established as a subsistence strategy by 1300 BCE. Ruminant milk proteins were identified in the dental calculus of most of the tested LBA Khövsgöl individuals, and all identified milk proteins originated from ruminants, specifically the Western dairy domesticates sheep, goat, and Bovinae. These findings suggest that neighboring WSH populations directly or indirectly introduced dairy pastoralism to local indigenous populations through a process of cultural exchange. Further research on other regional cultures in Mongolia, such as Chemurchek, Hemsteg, and Ulaanzuukh, is needed to determine if this pattern of cultural adoption observed among DSKC sites is broadly shared across other Bronze Age cultures throughout the Eastern steppe.

Bronze Age trade and cultural exchange are difficult to observe on the Eastern steppe, where mobile lifestyles and ephemeral habitation sites combine to make household archaeology highly challenging. Burial mounds are typically the most conspicuous features on the landscape, and thus much of Mongolian archaeology is dominated by mortuary archaeology. However, unlike WSH, whose kurgans typically contain a range of grave goods, many LBA mortuary traditions on the Eastern steppe did not include grave goods of any kind other than ritually deposited animal bones from horse, deer, and bovids. Given that Mongolian archaeological collections are typically dominated by human remains with limited occupational materials, the ability to reconstruct technological exchange, human–animal interaction, and secondary product utilization through the analysis of proteins preserved in dental calculus represents an important advance.

The 3,000-y legacy of dairy pastoralism in Mongolia poses challenging questions to grand narratives of human adaptation and natural selection (36). For example, despite evidence of being under strong natural selection (36), LP was not detected among LBA Khövsgöls, and it remains rare (<5%) in contemporary Mongolia even though levels of fresh and fermented dairy product consumption are high (35). Recent studies in Europe and the Near East have found that dairying preceded LP in these regions by at least 5,000 y, suggesting that LP may be irrelevant to the origins and early history of dairying (36). As a non-LP dairying society with a rich prehistory, Mongolia can serve as a model for understanding how other adaptations, such as cultural practices or microbiome alterations (37), may be involved in enabling the adoption and long-term maintenance of a dairy-based subsistence economy. Early herding groups in Mongolia present a historical counter-example to Europe in which WSH migrations resulted in cultural exchange rather than population replacement, and dairying was maintained for millennia without the introgression or selection of LP alleles.

Materials and Methods

Experimental Design. Based on an 850-km^2 archaeological survey of DSKC-associated burial mounds in Arbulag soum, Khövsgöl, Mongolia, we selected 22 burial mounds from 6 distinct burial mound groupings (A–F) for excavation and analysis (Fig. 1 and *SI Appendix*, sections 1 and 2 and Table S1). Bone and tooth samples from 22 individuals (11 femora, 11 teeth) were analyzed for ancient DNA, and 12 dental calculus samples from 9 individuals were analyzed for ancient proteins (*SI Appendix*, Table S2). Twenty-one individuals were successfully direct radiocarbon dated to ca. 1380–975 BCE (*SI Appendix*, section 3 and Table S2).

Ancient DNA Extraction, Library Construction, and Sequencing. DNA extraction and library construction was performed in a dedicated clean room facility at the Max Planck Institute for the Science of Human History in Jena, Germany following previously published protocols (38), including partial uracil-DNA-glycosylase treatment (19). Following screening, 20 samples with ≥0.1% endogenous content were enriched for 1.2 million informative nuclear SNPs (1240K) by in-solution hybridization (2, 3). Additionally, preenrichment libraries for two well-preserved samples (ARS008 and ARS026) were deep-sequenced to generate ∼3.3× genomes. All sequencing was performed using single-end 75-bp (for screening and enriched libraries) or paired-end 50-bp (for whole-genome sequencing of two preenrichment libraries) sequencing on an Illumina HiSEq 4000 platform following the manufacturer's protocols (*SI Appendix*, section 4).

DNA Sequence Data Filtering and Quality Assessment. DNA sequences were processed using the EAGER v1.92.50 pipeline (39). Adapter-trimmed reads ≥30 bp were aligned to the human reference genome using BWA aln/samse v0.7.12 (40) with the nondefault parameter "-n 0.01," and PCR duplicates were removed using dedup v0.12.2 (39). The first and last three bases of each read were masked using the trimbam function in bamUtils v1.0.13 (41). For each target SNP, a single high-quality base (Phred-scaled quality score ≥30) from a high-quality read (Phred-scaled mapping quality score ≥30) was randomly chosen from the 3-bp masked BAM file to produce a pseudodiploid genotype for downstream population genetic analysis. DNA damage was assessed using mapDamage v2.0.6 (42), and mitochondrial DNA contamination was estimated using Schmutzi (43). For males, nuclear contamination was estimated using ANGSD v0.910 (44) (*SI Appendix*, section 4).

Uniparental Haplogroup and Kinship Analysis. Mitochondrial haplogroups were determined by generating a consensus sequence using the log2fasta program in Schmutzi (43), followed by haplogroup assignment both by HaploGrep2 (45) and HaploFind (46). The Y haplogroup was determined using the yHaplo program (47). Genetic relatedness was estimated by calculating pairwise mismatch rate of pseudodiploid genotypes (48) (*SI Appendix*, section 4).

Population Genetic Analysis. Khövsgöl SNP data were merged with published ancient genome-wide data for the 1240K panel (1, 3, 4, 20–22, 25–28, 49–59) (Dataset S1). A comparative dataset of present-day individuals was compiled from published datasets either genotyped on the Affymetrix Axiom Human Origins 1 array (HumanOrigins) or sequenced to high-coverage in the Simons Genome Diversity Project (20, 60–62) (*SI Appendix*, section 4). Intersecting with SNPs present in the HumanOrigins array, we obtain data for 593,124 autosomal SNPs across world-wide populations. Population structure was investigated by PCA as implemented in the smartpca v13050 in the Eigensoft v6.0.1 package (63) and by unsupervised genetic clustering using ADMIXTURE v1.3.0 (23) (*SI Appendix*, sections 4 and 5). The f_3 and f_4 statistics were calculated using the qp3Pop (v400) and qpDstat (v711) programs in the admixtools v3.0 package (24). For calculating the f_4 statistic, we added the "*f4mode: YES*" option to the parameter file. For admixture modeling, we used qpAdm v632 (20) in the admixtools v3.0 package (*SI Appendix*, sections 4 and 5).

Strontium Isotope Analysis. Strontium isotopes ($^{87}Sr/^{86}Sr$) measured from human and faunal tooth enamel ($n = 16$) and bone ($n = 5$) were analyzed at the University of Georgia Center for Applied Isotope Studies ($n = 17$) and the University of Florida Department of Geological Sciences ($n = 4$) using a thermo-ionization mass spectrometer (*SI Appendix*, section 6).

Dietary Analysis in Contemporary Khövsgöl, Mongolia. Up to 6 d of weighed diet records were collected from 40 subjects ($n = 231$ total person-days) randomly sampled from the rural soum of Khatgal and the provincial center of Mörön in June 2012 and January 2013 by trained medical students from the Mongolian National University of Medical Sciences and Ach Medical Institute. Nutrient consumption was determined using a purpose-built food composition table (64), which we appended with unpublished food composition data from the Mongolian University of Science and Technology and the Mongolian Public Health Institute, as well as published data from the United States and Germany (65, 66) (*SI Appendix*, section 7). Contemporary dietary data were collected under Harvard Institutional Review Board Protocol #21002.

Protein Extraction, Digestion, and LC-MS/MS. Ancient protein analysis was performed in a dedicated clean room facility at the Max Planck Institute for the Science of Human History following recommended guidelines (32). Dental calculus was decalcified in 0.5 M EDTA, and proteins were extracted and trypsin-digested using a modified low-volume Filter-Aided Sample Preparation protocol (67). The resulting peptides were analyzed by LC-MS/MS using a Q-Exactive HF mass spectrometer (Thermo Scientific) coupled to an ACQUITY UPLC M-Class system (Waters) at the Functional Genomics Center Zurich, according to previously published specifications (25). Extraction blanks and injection blanks were processed and analyzed alongside experimental samples (*SI Appendix*, section 8).

Spectrum Analysis, Data Filtering, and Authentication. Raw spectra were converted to Mascot generic files using MSConvert using the 100 most intense peaks from each spectrum, and MS/MS ion database searching was performed using Mascot software (v2.6; Matrix Science) with the databases SwissProt (version 2017_07; 555,100 sequences) and a custom dairy database consisting of 244 dairy livestock milk protein sequences obtained from the National Center for Biotechnology Information GenBank. Before analysis, an error-tolerant search was performed (*SI Appendix*, Table S12) to identify common variable modifications (deamidation N, Q; oxidation M, P). Reversed sequences for each entry in both databases were added to perform downstream FDR calculations in R. Peptide tolerance was set at 10 ppm, with an MS/MS ion tolerance of 0.01 Da, and the data were filtered to only include peptides with an *E*-value ≤ 0.001 and proteins supported by a minimum of two peptides (*SI Appendix*, section 8). Peptides identified as matching milk proteins were tested for taxonomic specificity using BLASTp against the National Center for Biotechnology Information nr database and aligned to protein sequences of known dairy livestock. Modeling of β-lactoglobulin coverage was rendered using VMD v.1.9.4a7, and an additional level of protein identification confirmation was performed by comparing the observed ancient milk peptides to modern proteotypic peptides using the R package ggplot2 (68) with published data for bovine β-lactoglobulin obtained from the Peptide Atlas (33) (*SI Appendix*, section 8).

Phenotype-Associated SNPs. Genotype likelihoods for phenotype-associated SNPs were calculated using the UnifiedGenotyper program in the Genome Analysis Toolkit v3.5 (69) (*SI Appendix*, section 9).

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4. Manuscript B

"Dairy pastoralism sustained eastern Eurasian steppe populations for 5000 years"

Shevan Wilkin, Alicia Ventresca Miller, William Taylor, Bryan K. Miller, Richard Hagan, Christian Trachsel, Jonas Grossmann, Abigail Ramsøe, Ludovic Orlando, Mark Horton, Philipp Stockhammer, Erdene Myagmar, Nicole Boivin, Christina Warinner, Jessica Hendy

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The second project expands upon the resulting data from the first by analysing the dental calculus from 32 individuals across Mongolia spanning between the Early Bronze Age and the Mongol Empire (ca. 3000 BC - 1500 AD), and has the following specific outcomes:

- To locate the earliest dairy consuming culture group in Mongolia
- To classify the range of dairy species exploited in early and imperial Mongolia
- To identify the earliest use of horse milk on the eastern Eurasian steppe

Author contributions: Shevan Wilkin, William Taylor, Christina Warinner, Nicole Boivin, and Jessica Hendy designed the research plan; Choongwon Jeong, Shevan Wilkin, Richard W. Hagan, Christian Trachsel, Jonas Grossmann, Abigail Ramsøe, Erdene Myagmar, Jessica Hendy, and Christina Warinner performed research; Richard W. Hagan, Christian Trachsel, Jonas Grossmann, and Ashely Scott contributed analytic tools; Nicole Boivin contributed personnel and material resources; Shevan Wilkin, William T.T. Taylor, Richard W. Hagan, Christian Trachsel, Jonas Grossmann, Jessica Hendy, and Christina Warinner analyzed data; and Shevan Wilkin and Jessica Hendy wrote the paper. In total, Shevan Wilkin contributed to 80% of this research.

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Dairy pastoralism sustained eastern Eurasian steppe populations for 5,000 years

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Dairy pastoralism is integral to contemporary and past lifeways on the eastern Eurasian steppe, facilitating survival in agriculturally challenging environments. While previous research has indicated that ruminant dairy pastoralism was practiced in the region by circa 1300 BC, the origin, extent and diversity of this custom remain poorly understood. Here, we analyse ancient proteins from human dental calculus recovered from geographically diverse locations across Mongolia and spanning 5,000 years. We present the earliest evidence for dairy consumption on the eastern Eurasian steppe by circa 3000 BC and the later emergence of horse milking at circa 1200 BC, concurrent with the first evidence for horse riding. We argue that ruminant dairying contributed to the demographic success of Bronze Age Mongolian populations and that the origins of traditional horse dairy products in eastern Eurasia are closely tied to the regional emergence of mounted herding societies during the late second millennium BC.

In contemporary central and eastern Eurasia, mobile dairy-based pastoralism is a key subsistence practice for many people[1]. Much of the eastern Eurasian Steppe is covered by dryland grasses which, while challenging for grain agriculture, can sustain large meat and dairy-producing herds[2,3]. Across the steppe, dairy is a staple food and the product of rich culinary traditions. In the Mongolian countryside, fresh, fermented, processed and distilled dairy products provide a major source of hydration and up to 50% of summer caloric intake[1,4]. Moreover, milk provides a protein- and fat-rich dietary component, while the processing of milk into dairy products enables the creation of a storable and transportable food source. In Mongolia today, dairy livestock, including sheep, goat, horse, cow, yak, reindeer and camel, are exploited for milk, meat, traction and transport across diverse environmental niches.

The adoption of dairy into adult human diets was a major transition in prehistoric subsistence[5]. In western Eurasian contexts, biomolecular approaches have been extensively applied to investigate the archaeological antiquity of a dairy diet. Following their initial domestication in Southwest Asia, cattle (*Bos taurus*), sheep (*Ovis aries*) and goats (*Capra hircus*) spread eastwards across the Eurasian steppe into Central Asia[6]. Biomolecular evidence for dairy lipids has been identified in ceramics from Neolithic Anatolia[7] and eastern Europe[8], as well as Copper and Bronze Age Kazakhstan[9,10], indicating the potential spread of dairying out of Southwest Asia. As far east as the Tarim Basin of the Xinjiang region in northwestern China in the Middle Bronze Age, milk proteins have been identified in a woven basket[11] and pieces of well-preserved kefir cheese[12] (Fig. 1). In Bronze Age Mongolia, a recent study of human dental calculus from individuals across multiple sites in the northern Khövsgöl aimag identified milk proteins from sheep, goat and cattle[1]. Ancient DNA analysis of the same population found that almost all individuals were of predominantly local ancestry and only a single individual had over 10% of western steppe herder ancestry. This suggests that by the late second millennium BC, dairy pastoralism had been fully adopted by, or originated with, local northern populations, leaving an open question of when and how dairy subsistence arrived in this region.

Direct evidence into the timing and nature of pastoral economies in Mongolia from other datasets is exceedingly rare. On the eastern Steppe, the ephemeral nature of pastoral campsites and severe wind deflation in most contexts makes detecting occupational sites with direct information on subsistence economies challenging[13,14]. As a result, archaeologists have often been forced to form conclusions about local subsistence from materials found in ritual

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Fig. 1 | Ruminant and equine dairying in prehistoric Eurasia and contemporary Mongolia. a, Map of Eurasia showing major geographical features referred to in the text and sites where evidence of dairying has been previously found using proteomic approaches: (1) Khövsgöl[1], (2) Xiaohe[11], (3) Gumugou[10], (4) Subeixi[68], (5) Bulanovo[29], (6) Hatsarat[29], (7) Çatalhöyük West[69], (8) Tomb of Ptahmes[70], (9) Szöreg-C (Sziv Utca)[29] and (10) Olmo di Nogara[29]. Locations for the earliest evidence of ruminant dairying based on the presence of milk fats in ceramics are shown in blue[7] and the earliest evidence of horse dairying[9] are shown in pink. Details for each site included in this figure are referenced in Supplementary Table 3. **b**–**f**, Mongolian dairy products from Khövsgöl aimag: yoghurt starter culture, Khöröngö (Хөрөнгө) (**b**); curd from reindeer milk, 'kurd' (**c**); dried curd from mixed yak and cow milk, aaruul (ааруул) (**d**); clotted cream from mixed yak and cow milk, öröm (өрөм) (**e**); and fermented horse milk, airag (айраг) (**f**). **g**, Dairying ritual from Dundgobi aimag, Mongolia blessing the first horse airag production of the season. Credit: Photograph **c** provided by Matthäus Rest; photograph **d** provided by Jessica Hendy; and all others provided by Björn Reichhardt.

human burials under stone monuments that dominate the Bronze Age archaeological landscape and occasionally include satellite animal burials. Specific features of burial mounds (stone type, shape and ringed fences) can be used to identify interred individuals into different culture groups as mound construction styles changed alongside evolving cultural traditions in Bronze Age Mongolia[15,16]. Prior to the Bronze Age, before the presence of constructed stone burial mounds, there are very few uncovered occupation or ritual sites, and pre-Bronze Age subsistence strategies are not well understood. However, it is assumed that Neolithic subsistence strategies included hunting, gathering and fishing, although the possibility of pastoralism should not be completely discounted[14]. Human burials associated with Afanasievo and Chemurchek culture groups (circa 3000–2500 BC) contain faunal remains of ovicaprid, bovine, equid and dog remains[15,17–19], yet it is unclear whether the remains of each were of a domesticated variety or their wild relatives, such as *Ovis ammon* and *Capra ibex* in the case of ovicaprids[20]. Later Bronze Age (1500–800 BC) campsites containing ruminant and equine remains suggest the dietary consumption of horses and cattle, as well as sheep and/or goats as part of a fully pastoral economy[21]. Satellite burials containing multiple animals surrounding ritual human internments attest to pastoral culling and herd management patterns of sheep, goat, cattle and horses during this period[22,23]. By 1200 BC, remains of domesticated horses became almost ubiquitous at ritual burials sites in Northern Mongolia, with some of the crania showing evidence for equine dentistry and horse bridling and riding[24,25].

In later time periods, written records from neighbouring regions, such as China and the Middle East, as well as within Mongolia, document the importance of ruminant and equine dairy in day-to-day subsistence, particularly the consumption of fermented horse milk as early as the Xiongnu Empire (circa 200 BC to AD 100)[26] along with camel milk by the Mongol period (circa AD 1206–1398)[27,28]. Even though many archaeologists and historians assume milk had been included in ancient steppe diets, little direct evidence has been available about where and when specific animal species were first exploited for dairy on the steppe east of the Altai Mountains.

The analysis of ancient proteins extracted from ancient human dental calculus (calcified dental plaque or tooth tartar) has been established as an approach for detecting milk consumption in past individuals[1,29–31]. Differences in amino acid sequences between taxa enable the detection of the livestock species or 'zooarchaeology by proxy'—a method to detect past animal use by the analysis

of human remains alone. Here, we apply the proteomic analysis of ancient dental calculus to 32 individuals spanning from the late Neolithic through the Middle Ages to characterize the antiquity and species diversity of ruminant and equine dairying in Mongolia. We report the earliest direct evidence for dairy consumption in East Asia (east of the Altai Mountains), finding that ruminant dairy consumption was a feature of ancient diets in Mongolia from its initial pastoral occupation, circa 3000 BC and occurred in association with archaeological sites linked to western steppe cultures. We show that ruminant dairying became widespread by the Middle Bronze Age (1800–1200 BC) and from 1200 BC we observe the onset of horse milk consumption, perhaps the progenitor of the alcoholic drink airag, in tandem with the first evidence for mounted horseback riding and highly mobile economies—a tradition that remained important through the great nomadic empires and into the modern era.

Results

Milk proteins were identified in 72% of individuals analysed (23 of 32 individuals, Table 1), indicating the widespread consumption of dairy foods across multiple time periods in prehistoric and historic Mongolia. Specifically, we detected evidence of the milk proteins β-lactoglobulin (BLG I and II), α-S1-casein, kappa-casein, α-lactalbumin and β-casein, lysozyme C and peptidoglycan recognition protein 1. BLG was the most frequently detected milk protein, a pattern consistent with previous observations from ancient dental calculus[1,32]. We observe evidence of milk consumption from diverse taxa across multiple environmental zones within Mongolia (Fig. 2). Of the seven dairy livestock species used in contemporary Mongolia, we identified milk peptides from goat, sheep, cattle, horse and camel but did not detect peptides that could be assigned specifically to reindeer or yak milk.

We find evidence of milk proteins in the earliest directly dated individual in our sample set, AT-26 (3316–2918 cal BC; 2σ range) at the Afanasievo burials of Shatar Chuluu, the earliest known mounded burial features associated with pastoral economies in the territory of Mongolia. Specifically, we observe peptides deriving from a taxonomically ambiguous region of the milk whey protein BLG, where the species can be assigned as a bovid in Bovinae subfamily (cow, yak, bison and water buffalo) or *Ovis* genus (sheep), making a more specific taxonomic assignment for this individual's milk consumption more challenging. In the two individuals associated with Chermurchek culture at the sites of Khundii Gobi (2886–2577 cal BC; 2σ range) and Yagshiin Khuduu (2567–2468 cal BC; 2σ range), we detect milk peptides matching to the subfamily Caprinae (sheep or goat) and the genus *Ovis* (sheep) from BLG and also α-S1-casein. At Khundii Gobi, these identifications are specific to sheep (with six BLG peptides identified) among others that are specific only to the subfamily Caprinae and the infraorder Pecora (all even-toed ruminant mammals). These results align with recent archaeofaunal data from the early second millennia BC, which suggest a significant role for sheep in the prehistoric economy of pastoral occupants of the Mongolian Altai[33].

In the Middle Bronze Age (circa 1800–1200 BC) the consumption of ruminant dairy milk can be seen in four of seven individuals analysed in the central eastern site of Ulaanzuukh and in seven of nine individuals in the previously published Khövsgöl sites in northern central Mongolia. Ulaanzuukh individuals date to slightly earlier than the Bronze Age burial sites analysed in Jeong et al.[1], where evidence of ruminant milk consumption was found in individuals associated with slope burials and khirigsuur ritual monuments. At Ulaanzuukh, BLG is the most frequently recovered protein across all samples, with peptides from α-S1-casein and kappa-casein (proteins that are associated more with milk curds than milk whey) identified to a lesser extent. The dental calculus from the three Ulaanzuukh individuals who did not show evidence of dairy consumption showed a poor level of protein preservation, with a general absence of typical salivary and bacterial proteins reported previously in dental calculus studies[34], which may suggest that an absence of evidence for milk consumption in these individuals could be due to overall poor biomolecular preservation.

At Bronze Age sites dating to after 1200 BC, we found evidence for dairy consumption in all four individuals tested. We identified ruminant milk peptides from the same two proteins (BLG and α-S1-casein) identified in the previous time period, as well as from two additional proteins: α-lactalbumin and β-casein. In addition to ruminant milk proteins, we also detected the first palaeoproteomic evidence of horse (*Equus*) milk, including horse-specific peptides from BLG I and II (horse BLG is derived from two paralogous genes) in two of four individuals. An individual from the site of Shunklai Uul (circa 1000 BC), in central Mongolia, had 126 peptide spectral matches (PSMs) from ruminant BLG, another 50 from horse BLG I and II (Fig. 3) and an additional nine from α-S1-casein, β-casein and α-lactalbumin.

Of the three Early Iron Age individuals (800–400 BC), two from the northwestern Mongolia site of Chandman Mountain and another from Dartsagt (north-central Mongolia), all showed moderate to high preservation and contained an abundance of human and oral microbiome proteins. Two of these contained no robustly identified milk peptides; in contrast, one individual from Chandman Mountain contained an abundance of PSMs to milk proteins. During this period, BLG peptides specific to both sheep and goat were detected, along with others that can be assigned to the higher taxonomic orders of Caprinae and Bovidae. Also, we find peptides derived from casein and α-lactalbumin specific to caprines, and those from both BLG I and II specific to equine milk.

In the Late Iron Age, during the tenure of the Xiongnu Empire (circa 200 BC to AD 300), calculus from each of the three individuals studied contained evidence for dairy consumption. One individual had PSMs specific to only horse BLG, while the second had *Bos*-specific BLG and caprine α-S1-casein peptides. The third individual had ruminant casein and whey proteins (BLG and α-S1-casein) as well as lysozyme C peptides specific to *Equus*. In the post-Xiongnu period, a single individual archaeologically classified as Turkic era did not show any evidence of milk consumption.

Nine of the eleven Mongol Empire individuals showed evidence for the consumption of dairy, with many individuals showing evidence for the consumption of milk from multiple species. For example, one individual showed evidence for the consumption of ruminant, equine and camel milk, whilst a further five individuals showed evidence for the consumption of both ruminant and horse dairy products (Fig. 3). During this period, we observed the first evidence for the consumption of camel milk through the detection of peptidoglycan recognition protein 1 (UniProtKB: Q9GK12), an immune protein that has been isolated from modern camel milk.

Deamidation of glutamine and asparagine has been proposed as a marker of taphonomic degradation in ancient proteins[35]. We applied an analysis of bulk deamidation using a previously published approach[36] to five individuals from this study to examine potential patterns of archaeological degradation in milk, which may suggest that the milk proteins in our samples are ancient, as they appear to be of similar deamidation levels as the human oral proteins in the calculus. All deamidation is reported in Supplementary Table 2. We observed that the older samples generally showed higher levels of deamidation. The milk-origin peptides retrieved from the early Bronze Age individuals showed an average of 23.9% glutamine deamidation, the Late Bronze Age 13.5% and the Mongol period 3.3%. The same pattern was observed in the deamidation of asparagine, with an average of 52.6% deamidation of milk-origin peptides in the Early Bronze Age and 32.9% in the Late Bronze Age. However, the milk peptides recovered from the Mongol period individual do not fit this pattern in terms of asparagine deamidation, with 48.3% deamidation (Supplementary Table 2).

Table 1 | Presence of dairy proteins by individual and archaeological site

Archaeological culture	Archaeological site	Individual ID	Calibrated radiocarbon date	Milk species/taxonomic group identified
Early Bronze				
Afanasievo	Shatar Chuluu	AT-26	3316–2918 BC	Bovinae/*Ovis**
Chemurchek	Khundii Gobi	AT-628	3310–2919 BC	*Ovis*, Bovinae/*Ovis*
Chemurchek/ Afanasievo	Khuurai Gobi	AT-635	2618–2487 BC	None detected
Chemurchek	Yagshiin Huduu	AT-590b	2567–2468 BC	*Ovis*, Bovinae/*Ovis**, Caprinae
Middle Bronze				
Ulaanzuukh	Ulaanzuukh	AT-823	1391–1209 BC	None detected
		AT-921	1412–1266 BC	*Capra, Ovis*, Bovidae
		AT-923	No date	Bovinae/*Ovis**
		AT-824	1402–1279 BC	None detected
		AT-769A	1608–1416 BC	Bovidae, Bovinae/*Ovis**
		AT-769B	1509–1439 BC	None detected
Late Bronze				
Baitag	Uliastai River, Central Terrace	AT-676	1277–1057 BC	*Equus, Capra, Ovis*, Bovinae
Deer Stone/ Khirigsuur	Berkh Uul	AT-905	1371–1121 BC	*Ovis, Capra*, Bovinae/*Ovis**
Slab Burial	Shunkhlai Uul	AT-233	1072–903 BC	*Equus, Capra, Ovis, Bos*
Undefined	Khoit Tsenkher	AT-398	1056–904 BC	*Capra*, Bovinae/*Ovis**
Early Iron				
Chandmani	Chandman Uul	AT-56	971–843 BC	*Equus, Capra, Ovis*, Bovinae
		AT-121	358–195 BC	None detected
Slab Grave	Dartsagt	AT-766	750–407 BC	None detected
Late Iron				
Xiongnu	Tamiryn Ulaan Khoshuu	AT-728	No date	*Equus*
Xiongnu	Duulaga Uul	AT-605	43 BC to AD 51	*Bos*, Bovinae/*Ovis**, Caprinae
Iron Age	Ulaanzuukh	AT-885	cal 96 BC to AD 61	Caprinae, Bovinae/*Ovis**
Early Medieval				
Turkic	Uliastain dood denj	AT-675	cal AD 650–765	None detected
Late Medieval (Mongol period)				
Mongol Empire	Sharga Uul	AT-701	No date	*Equus*
Mongol Empire	Del Khad	AT-775	No date	*Equus, Ovis, Camelus*
Mongol Empire	Zaraa Tolgoi	AT-271	No date	*Equus*, Bovinae/*Ovis**
Mongol Empire	Banzart Khairkhan	AT-846	No date	*Equus*, Bovinae/*Ovis**
Mongol Empire	Tahiltyn Khotgot	AT-360	No date	*Equus*
Mongol Empire	Burgaldain Khundii	AT-650	No date	*Equus*, Caprinae,
Mongol Empire	Ganzagad	AT-835	No date	*Equus, Capra*, Bovinae/Ovis*
Mongol Empire	Uguumur Tsuvaraa Uul	AT-549	No date	*Capra, Ovis*, Bovinae
Mongol Empire	Khoit Tsenkher, Tarvagatain Am	AT-354	AD 1158–1252	*Equus*, Caprinae, Bovidae
Mongol Empire	Kharkhorin	AT-512	No date	None detected
Mongol Empire	Mori Baridag	AT-800	No date	None detected

Radiocarbon dates are calibrated to a 2σ probability (Supplementary Table 6). Bovinae/*Ovis** identifications refer to the detection of a polymorphic amino acid position where in the identified peptide TPEVD(D/N/K)EALEK, D is specific to Bovinae, N is specific to *Ovis* and K is specific to *Capra*. Because asparagine (N) deamidates to aspartic acid (D), the presence of a D at this position cannot be unambiguously assigned to Bovinae or *Ovis*. References and details of archaeological site excavations can be found in the Supplementary Table 1. All identified dairy peptides per individual are detailed in Supplementary Dataset 2.

Discussion

Earliest evidence for dairying is associated with western steppe herder archaeological cultures. Our results demonstrate the oldest known evidence of dairy consumption in Mongolia and the eastern Eurasian steppe (circa 3000–2500 BC), in the form of Early Bronze Age Afanasievo- and Chemurchek-associated individuals in both central and western Mongolia (Table 1). Previous ancient DNA analysis of one of the individuals at Shatar Chuluu showing ruminant dairy proteins (AT-26) was shown to have a non-local mitochondrial haplogroup consistent with western steppe herder populations[37], supporting the interpretation of individuals associated with the Afanasievo culture as migrants from eastern Europe

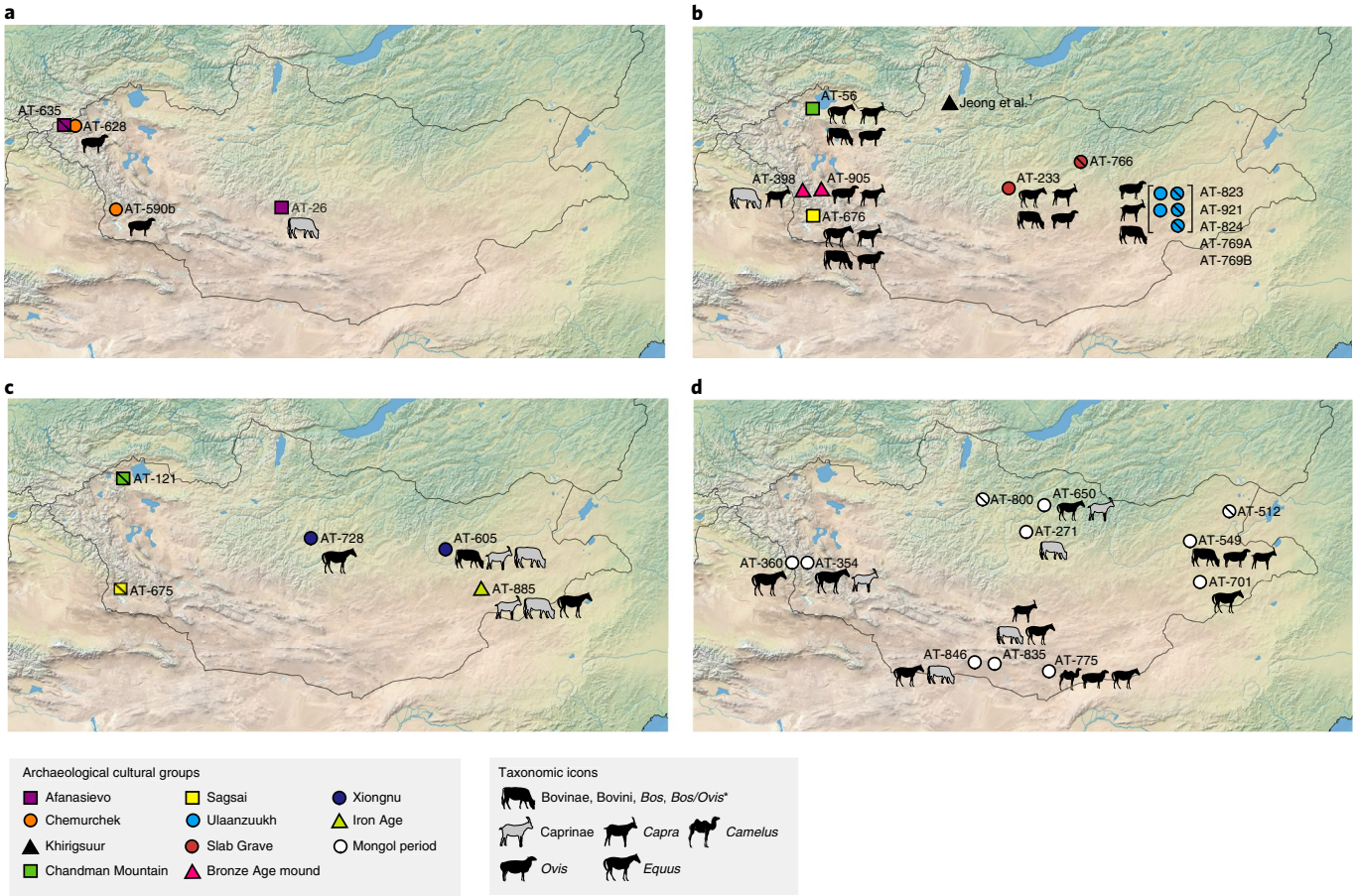

Fig. 2 | Mongolian dairy consumption by period. a–d, Maps showing changes in dairy consumption for Neolithic to Early Bronze Age (**a**), Middle–Late Bronze Age (**b**), Iron Age and Early Medieval (**c**) and Late Medieval (**d**). Archaeological site cultural affiliation is indicated by colours and symbols. Solid filled symbols indicate individuals with positive evidence of milk proteins, while symbols bisected with a diagonal line indicate individuals where no milk proteins were identified. Individuals of the same site are contained within brackets. Individual AT-923, associated with Ulaanzuukh, is not directly radiocarbon dated and is not included in this figure. Taxonomic icons only indicate the most specific taxa identified in a phylogenetic branch. The full list of dairy species identified for each individual is given in Table 1 and Supplementary Dataset 2. Data used in the creation of this figure are included in Supplementary Table 4.

via the Russian Altai[38–40] and a probable vector for the initial introduction of domestic animals into Mongolia[14]. The identification of ruminant milk proteins (*Ovis* and Bovinae/*Ovis*) supports the domestic nature of fauna found in these burial features and indicate that dairy pastoralism formed an important element of the subsistence base of these late fourth millennium BC transcontinental migrants. Most significantly, they suggest that human migrations associated with the expansion of the Afanasievo culture present a viable candidate for the initial introduction of dairy and domestic livestock into eastern Eurasia. In this study, the earliest individual in this dataset showed evidence of dairy consumption. To track the earliest instances of dairy consumption in the eastern Steppe, it would be necessary to analyse individuals from earlier time periods. This would be particularly informative to untangle the presence or absence of dairying before Yamnaya–Afanasievo migrations.

The antiquity of eastern steppe horse milk consumption. Temporal patterning in our protein results suggest that horse milk consumption played a key role in the emergence and proliferation of mobile pastoralism in Mongolia (Fig. 4). Today, horses play a vital role in traditional Central Asian pastoral lifeways, improving herd management as well as providing a primary source of meat and milk. Horseback herders can manage larger herd sizes. Horses can break through snow and ice to access the sustenance underneath, exposing grass oases for other animals in the herd[41–43]. We observe direct evidence of horse milk consumption on the eastern steppe, in the form of equine (*Equus*)-specific peptides from the milk whey proteins BLG (I and II) and lysozyme C in individuals associated with the Baitag and Slab Grave cultures in western Mongolia dated to the late second millennium BC (Table 1). In addition to the appearance of horses in dietary assemblages[21], this time period is linked with the proliferation of horses in ritual sites, the first direct archaeological evidence for horse bridling and riding[25,44], the first evidence for horse breeding and management[22,23], innovations in horse healthcare[24], an expanded use of dry intermontane grasslands[23,24,45] and the emergence of mobile, horse-facilitated pastoralism in eastern Eurasia. Our findings suggest that the incorporation of horses into dairy herds may have been closely linked to this multifaceted economic transformation in the use of horses[45].

Following the Bronze Age, direct proteomic evidence for horse milk consumption continues through the imperial Xiongnu and Mongol periods (Table 1), in agreement with extensive textual and zooarchaeological evidence underscoring their significance to historic economies. During the Iron Age, we identify horse milk proteins at Tamiryn Ulaan Khoshuu, a site within the heartland of the Xiongnu Empire[46,47]. Historical Chinese documents record that an assortment of dairy products were consumed during the Xiongnu period (circa 200 BC to AD 100), including dried curds from ruminant

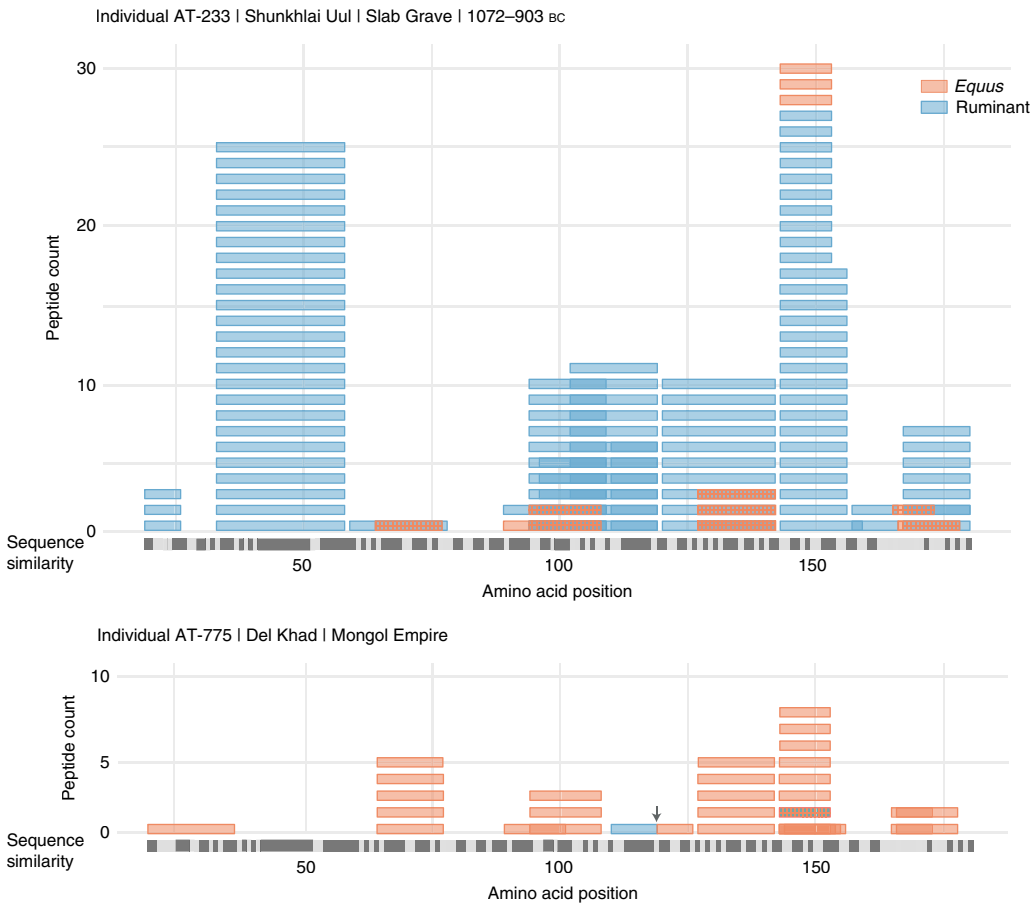

Fig. 3 | Alignment of observed BLG peptides for two individuals analysed in this study, showing the number of *Equus* and ruminant BLG peptides detected. For *Equus*, peptides from both *LGB1* and *LGB2* paralogues are shown (see Supplementary Table 5 for data associated with figure). Where peptides from these two taxa overlap, this has been indicated by a blue/orange cross-hatch pattern. The arrow in Individual AT-775 indicates two contiguous but independent peptides. Beneath each individual is a consensus sequence of *B. taurus* BLG (UniProt: P02754) and *E. caballus* BLG1 (UniProt: P02758) with dark grey indicating sequence identity and pale grey indicating sites with sequence differences.

milk (aaruul) but lao (horse alcohol) was the most consistently referenced dairy product and it held a prominent place in the cultural practices and identities of the steppe peoples[48–50]. By the Mongol period, horse milk consumption appears in among >80% (9 of 11) of tested individuals, a finding that matches historical accounts for widespread consumption of fermented mare's milk, known as airag (Mongolian) or koumiss/kymyz (Turkic languages)[27].

Horse milk differs from ruminant milks in important ways, in particular, it contains less curd protein (caseins) and much more lactose[41,51,52]. While the lower casein content makes it undesirable for producing most dried curd products (like aaruul, a staple of the traditional Mongolian diet), its high lactose content makes it highly suitable for making alcoholic beverages with ethanol contents as high as 10–12% (ref. [53]). Communal airag-drinking was and continues to be an important social activity (Fig. 1) and it has been frequently noted in both Mongolian and foreign historical texts. The cultural significance of airag continued throughout the Mongol period, with social gatherings at the time referred to as 'going to drink airag with people', and social rankings reinforced by how close one sat in relation to the pitchers of airag. Favoured associates were charged with serving the airag and with choosing the order in which airag was served at feasts[27]. At the Mongol capital of Kharkorum, a silver fountain was said to flow with fermented mare's milk, dispensing airag[54].

Identification of other milks and fermentation agents. In addition to cattle, sheep, goats and horses, domesticated Bactrian camels (*Camelus bactrianus*), yaks (*Bos grunniens*) and reindeer (*Rangifer tarandus*) are also milked in contemporary Mongolia; however, very little is known about the dairying history of these three species. Here we report protein evidence of camel milk consumption (together with horse and sheep milk) in a Mongol period individual buried in the Gobi Desert (Table 1), a habitat of Bactrian camels[55]. Although not unique to milk in other mammal species, the camel protein we identify here, peptidoglycan recognition protein 1 (PRP1), is an important component of camel milk whey[56,57]. Mongol period historical accounts, such as the twelfth-century Secret History of the Mongols[27], and historic accounts from foreign travellers into the empire, such as Marco Polo[28], include stories of camels used for subsistence (milk, meat and blood) and transport of people and gers (round tents). While probably not the earliest instance of camel milk-drinking, the PRP1 protein data presented here provide biomolecular evidence for its consumption. Camel dairy use is not well understood and this finding provides an insight into exploitation of this historic traction animal for milk.

We did not detect any milk peptides specifically identified as yak or reindeer in this study. While yak is a commonly herded species in Mongolia today, their past use as a dairying animal is not well understood. Yak proteins are difficult to specifically identify due to their sequence similarity to other cattle species. Yak BLG, for example, differs from that of cattle at only a single amino acid across the entire BLG protein. While it is possible that some of the peptides assigned in this study to *Bos* or the higher taxonomies of Pecora,

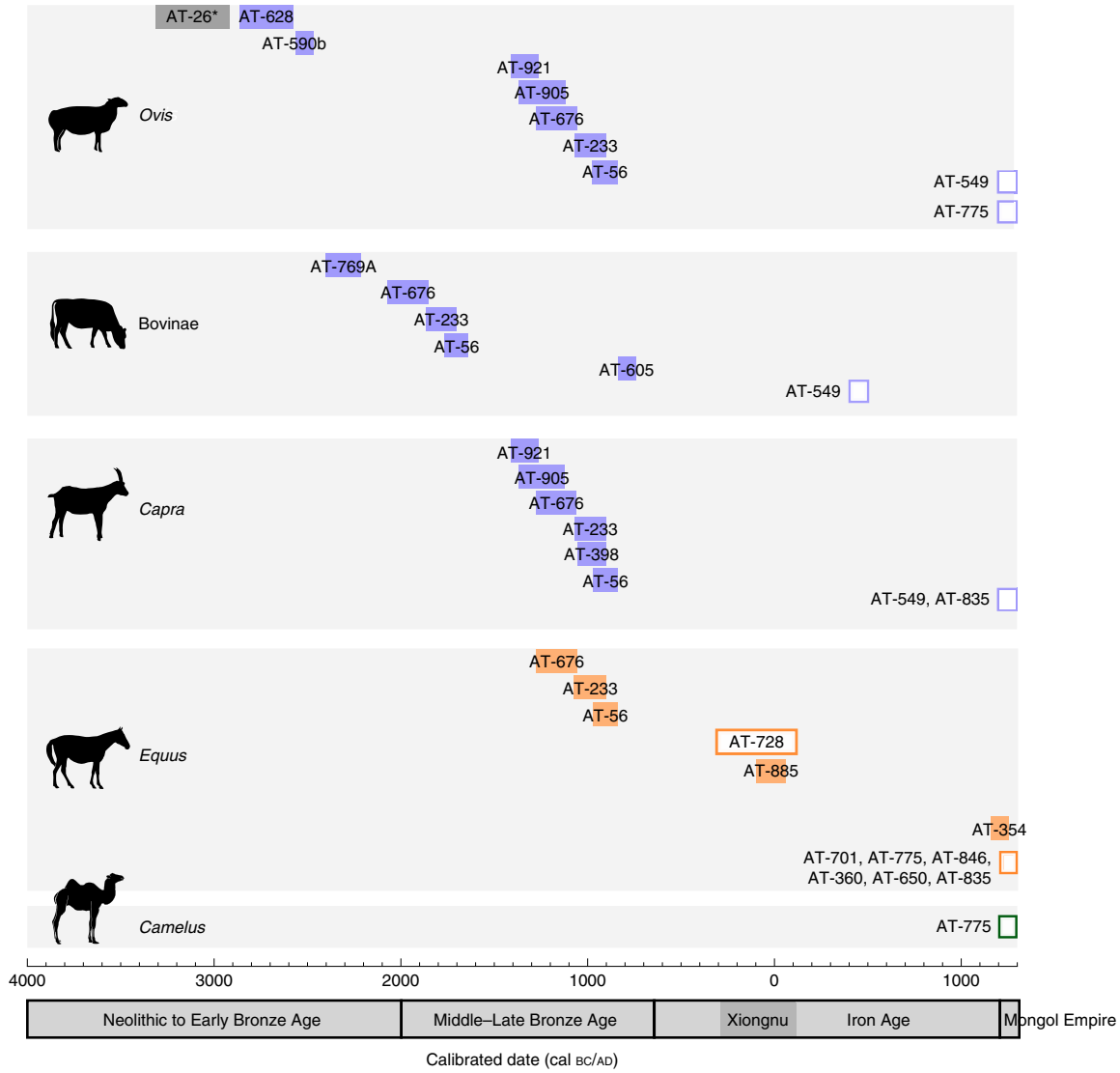

Fig. 4 | Timeline of evidence for the consumption of different livestock milk in prehistoric and historic Mongolia. Radiocarbon dates for each individual were calibrated using OxCal (OxCal v.4.3.2 Bronk Ramsey[66]; r:5 IntCal13 atmospheric curve[67]) and resulting radiocarbon probabilities were grouped by the taxa of dairy proteins identified in that individual (indicated by AT-numbers), with ruminant taxa (*Ovis, Capra* and Bovinae) indicated in purple, *Equus* indicated by orange and *Camelus* indicated by green. Dairy peptides identified in individual AT-26 (indicated with an asterisk) are specific to Bovinae/*Ovis*. Individuals without direct radiocarbon dates are indicated by unfilled boxes and are placed on the timeline based on the estimated time spans for the Xiongnu and Mongol Empires. For data used in this figure, refer to Supplementary Table 6.

Bovidae and Bovinae could have originated from domestic yak, we only observed cattle-specific variants when sufficient protein coverage enabled it. The absence of reindeer-specific milk consumption may be expected based on historical accounts on the use of this animal for dairying in this region. Contemporary reindeer herders in Mongolia do milk their animals but these families migrated from Siberia into northern Mongolia only in the last century[58,59].

In the present study, we did not identify any proteins specific to any bacterial taxa or other fermentation agents used in the processing of milk into other dairy products. Although processing agents were not found in the samples analysed in this study, it does not mean these were not used in ancient and historic Mongolia and future studies should continue to look for their presence alongside milk peptides.

These results provide the earliest evidence for dairy pastoralism in Mongolia's first herding societies, pushing back previous estimates of dairying in the Eastern steppe by more than 1,700 years and tracing pastoralist connections between the western and eastern steppes to the Early Bronze Age. These results show that within 5,000 years after the earliest evidence for dairying in the Near East, this practice and its associated animals had spread more than 7,000,000-km eastward to become a successful mode of subsistence on the Mongolian steppe. While the routes of this movement remain to be fully understood, our data suggest that dairy pastoralism, and in particular the emergence of horse riding and horse milking circa 1200 BC, provided the economic, political and social support necessary for the success of subsequent nomadic empires on the vast grasslands of Eurasia.

Methods

Sample collection. Dental calculus samples were collected from the Department of Anthropology and Archaeology at the National University of Mongolia (NUM) from 32 previously excavated individuals (Table 1, listed by NUM accession number; see Supplementary Table 1 for site details). Individuals were selected from archaeological sites assigned to time periods between the Neolithic and the Mongol period. Dental calculus was removed from the tooth using sterilized dental scalars and stored in Eppendorf tubes until extraction. Nitrile gloves were used during sample collection to avoid contamination from skin proteins. Samples were

exported to the Max Planck Institute for the Science of Human History under permission from the Ministry of Culture, Education, Science and Sports (export no. 10/413 (7b/52) was received on 2 February 2017, no. A0109258, MN DE 7 643). Protein extractions were conducted in a dedicated laboratory for the extraction of ancient proteins at the Max Planck Institute for the Science of Human History, Jena, using a filter-aided sample preparation protocol previously published in Jeong et al.[1]. Following protein extraction, digested peptides were stored at −80 °C before being analysed by liquid chromatography tandem mass spectrometry (LC–MS/MS) at the Functional Genomics Center Zürich, ETH/University of Zürich.

LC–MS/MS analysis. Mass spectrometry analysis was performed on a Q Exactive HF mass spectrometer (Thermo Scientific) equipped with a Digital PicoView source (New Objective) and coupled to an M-Class UPLC (Waters). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. Column temperature was 50 °C. For each sample 4 µl of peptides were loaded on a commercial ACQUITY UPLC M-Class Symmetry C18 Trap Column (100 Å, 5 µm, 180 µm × 20 mm, Waters) followed by ACQUITY UPLC M-Class HSS T3 Column (100 Å, 1.8 µm, 75 µm × 250 mm, Waters). The peptides were eluted at a flow rate of 300 nl min^{-1} by a gradient from 5 to 40% B in 120 min. Column was cleaned after the run by increasing to 98% B and holding 98% B for 5 min before re-establishing loading condition. Samples were acquired in a randomized order. The mass spectrometer was operated in data-dependent mode (DDA), acquiring a full-scan MS spectra (350−1,500 m/z) at a resolution of 120,000 at 200 m/z after accumulation to a target value of 3,000,000, and a maximum injection time of 50 ms followed by higher-energy collision dissociation fragmentation on the 12 most-intense signals per cycle. Higher-energy collision dissociation spectra were acquired at a resolution of 30,000 using a normalized collision energy of 28 and a maximum injection time of 50 ms. The automatic gain control was set to 100,000 ions. Charge state screening was enabled. Singly, unassigned and charge states higher than eight were rejected. Only precursors with intensity above 90,000 were selected for MS/MS. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 30 s and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on m/z 371.1012 and 445.1200.

Data analysis. To account for as much variation of milk-associated proteins as possible during MS/MS ion searches, a supplementary database of unreviewed milk protein sequences was curated from UniProtKB. As an additional source of dairy protein sequences, genomic data covering the two BLG genes in ancient horses generated by Gaunitz and colleagues[60] were translated into amino acid sequences, aligned with their respective modern sequences, and any putatively divergent proteins were concatenated to the supplementary database. In total, 244 additional accessions from UniProtKB and four putatively divergent BLG sequences from ancient horse genomes were added (Supplementary Dataset 1).

Peak lists were generated from raw files by selecting the top 100 peaks using MSConvert from the ProteoWizard software package v.3.0.11781 (ref. [61]). Each sample was searched using Mascot[62] (v.2.6.0) against Swiss-Prot in combination with the curated milk protein database (Supplementary Dataset 1). Results were exported from Mascot as csv files and further processed through an internally created tool, MS-MARGE[63] to estimate the validity of peptide identifications and summarize the findings. MS-MARGE is an R script that relies on an Rmarkdown file to generate the following: an HTML report summarizing the search (an example is given in the Supplementary Information file), a csv file containing confidently identified PSMs and a FASTA file of confidently identified peptide sequences. As input, MS-MARGE accepts a csv file exported from a Mascot MS/MS ion search against an amino acid database containing decoyed sequences with the Group Protein Families option turned off. Additionally, two parameters can be provided: an expected value (e-value) cutoff and a minimum number of peptides to support an identification (default 0.01 and 2, respectively). To estimate false discovery rate (FDR) at the PSM and protein level, MS-MARGE counts the number of decoy hits after filtering for e-value and minimum peptide support, and divides this value by the number of target hits minus the number of decoys. The resulting value is multiplied by 100 to provide an estimate of FDR as a percentage. We aimed for a protein FDR of under 5% and a peptide FDR of under 2% (Supplementary Dataset 2). A minimum of two individual PSMs were required for specific protein identifications and only peptides with an e-value below 0.01 were accepted. After filtering criteria were applied, we observed a range of variation in the numbers of proteins identified, with samples ranging from 2 to 209 confidently identified protein families.

Deamidation levels in five samples (AT-628, AT-590b, AT-26, AT-233 and AT-835) were calculated to assess authenticity using a previously published approach[36] (Extended Data Fig. 1 and Supplementary Table 2). These samples were specifically chosen to verify the antiquity of the oldest samples in this study, as well as a more recent sample from the Mongol period for comparison of deamination patterns. The raw MS/MS files for each were run through MaxQuant[64] v.1.6.2.6a against the previously described milk database and against the human proteome. Settings including a semitryptic search strategy allowing for a maximum of two missed cleavages were used, and the score cutoff for modified and unmodified peptides was set to 60 with no correction for FDR. Carbamidomethyl (C) was added as a fixed modification, while variable modifications included: oxidation (M), acetyl (protein N-term), deamidation (NQ), Gln-pyro-Glu (N-term E), Glu-pyro-Glu (N-term E), phospo (ST) and hydroxyproline. The deamidation levels of all milk proteins were averaged per sample. All identified peptides, including their posttranslational modifications, are reported in Supplementary Dataset 2.

Radiocarbon dating. A total of 26 bones and teeth from 24 individuals were radiocarbon dated at two research facilities, the Oxford Radiocarbon Accelerator Unit (ORAU, laboratory code OxA) and the Groningen Radiocarbon Laboratory (laboratory code GrM). The ORAU followed routine pretreatment and measurement procedures[65]. In brief, 200–600 mg of bone or dentine was drilled using a hand-held dentist drill and collagen was extracted through a series of chemical steps that involved immersion in HCl, removal of humic acids using NaOH and removal of adsorbed CO_2 via a final HCl wash. Only four of the samples prepared at the ORAU (OxA-36230, OxA-36231, OxA-36232 and OxA-36233) underwent ultrafiltration using Vivaspin ultrafilters, due to initial indications of poor collagen perseveration. Extracted collagen was frozen overnight and lyophilized. Between 2 and 5 mg of collagen was combusted in an elemental analyser (EA) and its C and N stable isotopes were measured at an isotope ratio mass spectrometer (IRMS) instrument linked to the EA, before excess gas CO_2 was collected, graphitized and measured at a High Voltage Engineering Europa (HVEE) accelerator, alongside blanks and standards. These were used for contamination calculation and final correction of the data. For these samples, collagen yields ranged greatly from 0.8% to 17.8%, C:N ratios of the extracted collagen fall within expected ranges (3.2–3.4), with the exception of OxA-36233 (C:N = 3.6), and percentage C in the combusted collagen was 37–46%. The measurements are reported in radiocarbon years before present (BP), where BP is AD 1950. Samples from Oxford were calibrated using OxCal v.4.3.2 (ref. [66]) and an IntCal13 atmospheric curve[67].

For radiocarbon dates processed at the Groningen Radiocarbon Laboratory, samples were decalcified over at least a 24-h period using mild acid (HCl, 2–4% w/vol; room temperature) at the Center for Stable Isotope Research at the University of Groningen. For each sample still not fully decalcified, the solution was refreshed, removing and storing soft portions separately in demineralized water until further preparation. Soft and pliable fragments were rinsed thoroughly with demineralized water. Extracts were then exposed to NaOH (1%, ~30 min) to eliminate humic acids, rinsed to neutrality and treated once more with acid (HCl, 4% w/vol, 15 min). The raw collagen fraction was denatured to gelatin in acidified demineralized water (pH 3) at 80 °C for 18 h. Before drying, the dissolved gelatin was filtered through a 50-µm mesh to eliminate any remaining foreign particulates and the crystalline collagen scraped from the glass. Approximately 4-mg aliquots of the reduced carbon fraction were then weighed into tin capsules for combustion in an EA (IsotopeCube NCS, Elementar). The EA was coupled to an IRMS (Isoprime 100), allowing the $\partial^{13}C$ value of the sample to be measured, as well as a fully automated cryogenic system to trap CO_2 liberated on combustion. After run completion, the individual reaction vessels were transferred to a graphitization manifold, where a stoichiometric excess of H_2 gas (1:2.5) was added and the CO_2 gas reduced to graphite over an Fe(s) catalyst. The graphite samples were then pressed and the radioisotopic ratio determined on a MICADAS accelerator mass spectrometer. Samples from Groningen were calibrated using OxCal v.4.3.2 (ref. [66]) and an IntCal13 atmospheric curve[67].

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Raw and processed MS/MS data from blanks, instrument washes and samples are available to download via the PRIDE partner repository under accession codes PXD014730 and PXD014730. The authors declare that all other data supporting the findings of this study are available within the paper and its supplementary information files. We have commissioned a Mongolian translation of this manuscript, available at https://natureecoevocommunity.nature.com/manage/posts/59870-mongolian-translation-of-dairy-pastoralism-sustained-eastern-steppe-populations-for-5000-years/edit.

Code availability

MS-MARGE, an R script used to estimate the validity of peptide identifications and summarize the findings is available for use via Bitbucket: https://bitbucket.org/rwhagan/ms-marge/src/master/. The custom dairy database used to analyse the data in this study is available to download via the York Research Database at https://doi.org/10.15124/589742eb-287a-4576-a00a-30df33d9f52c.

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Author contributions

S.W., W.T.T.T., N.B., C.W. and J.H. designed the research plan. S.W., M.B., S.G., M.H., S.U., E.M. and J.H. assessed archaeological collections and performed osteological assessments and subsampling. S.W., R.W.H., A.R., C.T., P.N. and J.H. performed laboratory work, mass spectrometry work and data analysis. R.W.H., A.S., A.R., C.T., J.G., P.W.S. and C.W. contributed to the development of data analysis tools. S.W., A.V.M., W.T.T.T., B.K.M., S.U., L.O., E.M., C.W. and J.H. contributed to archaeological data interpretation. J.H. and S.W. generated the figures. S.W. and J.H. wrote the paper with input from W.T.T.T., L.O. and C.W. and final approval from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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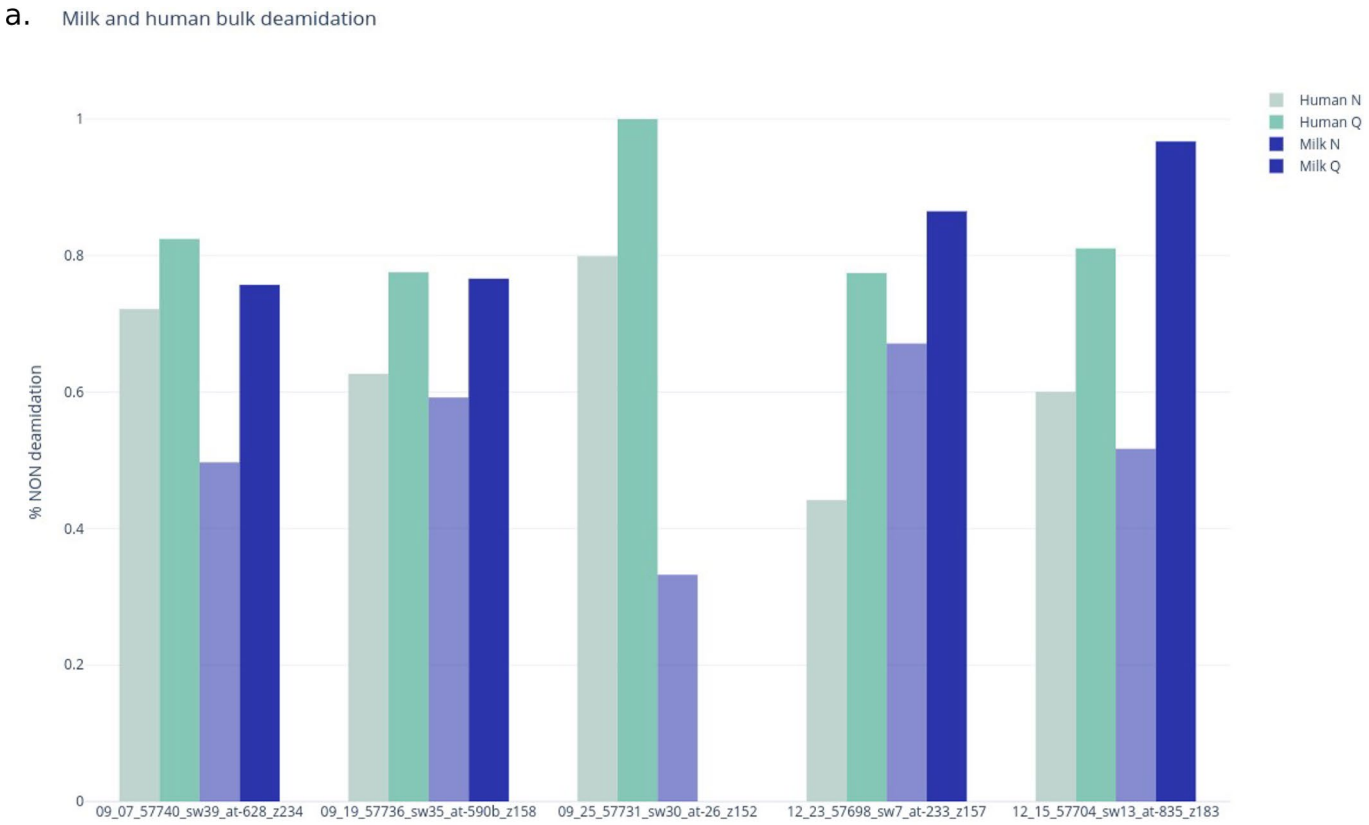

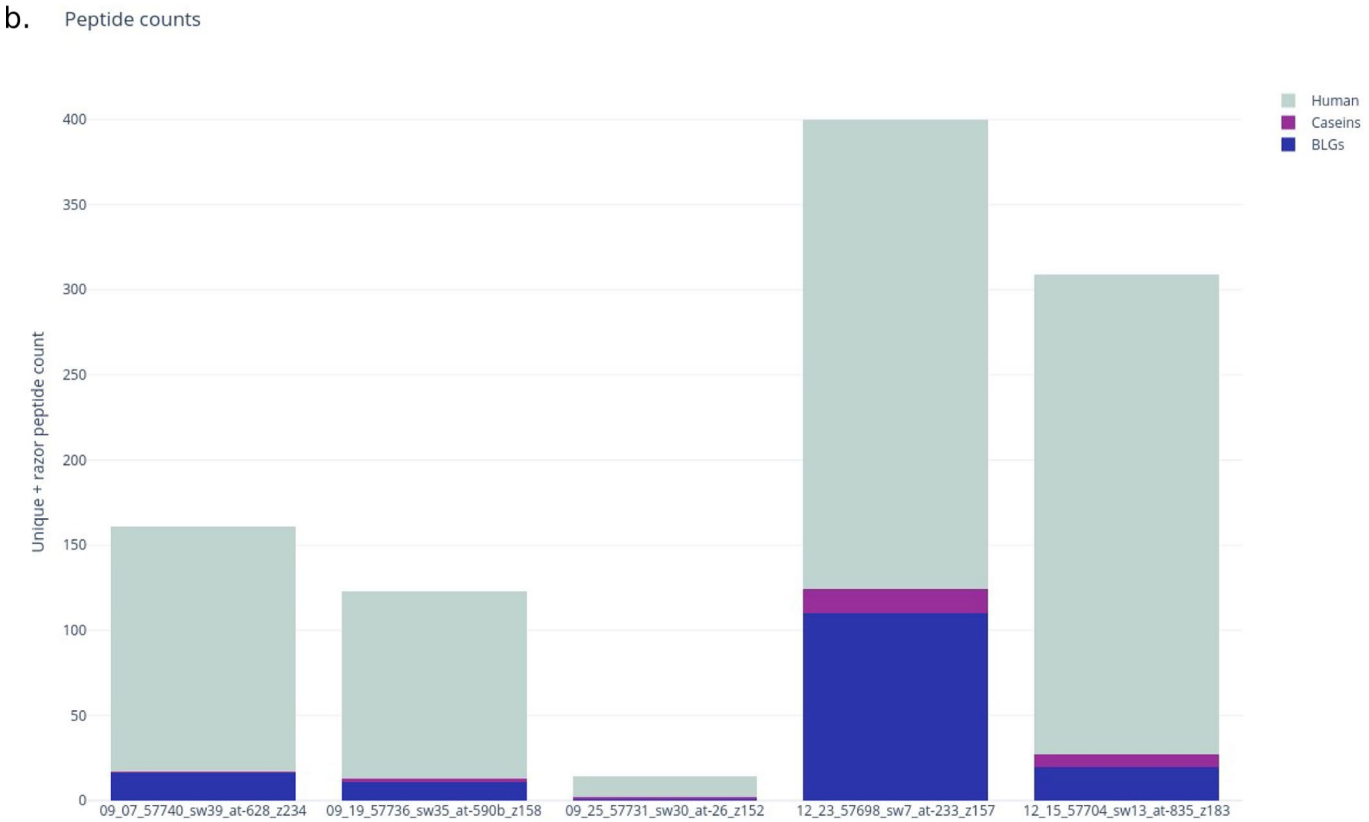

Extended Data Fig. 1 | Milk and bulk deamidation and peptide counts.

5. Manuscript C

"Economic Diversification Supported the Growth of Nomadic Mongolian Empires"

Wilkin, Shevan, Alicia Ventresca Miller, Bryan K. Miller, Robert Spengler, William T. T. Taylor, Ricardo Fernandes, Maddy Bleasdale, Jana Zech, Erdene Myagmar, Nicole Boivin, Patrick Roberts.

This project was published in *Scientific Reports* in March 2020

Project 3 explores the first and continued use of C_4 plants (millet) in Mongolia through stable isotope analysis of human bone collagen and dental bioapatite spanning between the Neolithic and Mongol periods (4500 BCE – 1400 CE).

- Measure range of carbon and nitrogen stable isotope values for human bone collagen and the range of carbon stable isotope values for human tooth enamel in each time period in Mongolia
- Identify when C_4 plants became a primary part of eastern steppe diets
- Investigate how dietary economies changed during times of empire and whether characterization as specialised pastoralists is appropriate

Author contributions: Shevan Wilkin, William Taylor, Nicole Boivin, and Patrick Roberts designed the research plan; Shevan Wilkin, Alicia Ventresca Miller. Bryan K. Miller, Richard W. Hagan, Ricardo Fernandes, Madeleine Bleasdale, Erdene Myagmar, and Patrick Roberts performed research; Nicole Boivin contributed personnel and material resources; Shevan Wilkin, Alicia Ventresca Miller, Bryan K. Miller, William Taylor, Ricardo Fernandes, Richard W. Hagan, analysed data; and Shevan Wilkin, Alicia Ventresca Miller, Bryan K. Miller, and Patrick Roberts wrote the paper. In total, Shevan Wilkin contributed to 80% of this research.

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Economic Diversification Supported the Growth of Mongolia's Nomadic Empires

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Populations in Mongolia from the late second millennium B.C.E. through the Mongol Empire are traditionally assumed, by archaeologists and historians, to have maintained a highly specialized horse-facilitated form of mobile pastoralism. Until recently, a dearth of direct evidence for prehistoric human diet and subsistence economies in Mongolia has rendered systematic testing of this view impossible. Here, we present stable carbon and nitrogen isotope measurements of human bone collagen, and stable carbon isotope analysis of human enamel bioapatite, from 137 well-dated ancient Mongolian individuals spanning the period c. 4400 B.C.E. to 1300 C.E. Our results demonstrate an increase in consumption of C_4 plants beginning at c. 800 B.C.E., almost certainly indicative of millet consumption, an interpretation supported by archaeological evidence. The escalating scale of millet consumption on the eastern Eurasian steppe over time, and an expansion of isotopic niche widths, indicate that historic Mongolian empires were supported by a diversification of economic strategies rather than uniform, specialized pastoralism.

Mongolian empires, such as the Xiongnu and Mongols, are some of the most renowned imperial entities in public and academic thought. This is, in part, due to their historical portrayal as highly mobile, predatory horseback polities with a specialized dairy and meat-based economy[1–4], an image that is perpetuated in cinema, novels, and documentaries alike. While such stereotypes likely arose from the hyperbolized accounts of neighboring adversaries, starting with the Han, who fought against the Xiongnu[5], they have persisted and now pervade academic evaluations of the economic basis of these ancient peoples. The modern economic focus on pastoralism in rural areas of Mongolia today is frequently viewed as a relic of the past and has been drawn upon to interpret the often-fragmentary archaeological record of this region[6,7], although ethnoarchaeological approaches often ignore the role of urban markets and motorized transport in modern mobile pastoralism. The view of uniformly specialized pastoral economies has also furthered the scholarly fascination with historical Mongolian populations, resurrecting the long-standing question of whether an empire can meet the costs and challenges of long-term political and economic organization in the absence of grain surpluses[6,7].

Empires are, however, inherently complex and, by definition, extend their control over multiple societies, cultures, and economies, as well as heterogeneous landscapes[8–10]. Crop surplus has traditionally been viewed as an essential component of stable political entities and complex imperial food production and procurement systems are often over-simplified by historians and archaeologists, leading to their characterization as single-resource systems (such as maize for the pre-Columbian empires of South America). Refined analyses generally reveal diverse and dynamic economies supporting imperial expansions, which draw together a variety of food sources[11].

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Time Period	Date Range	Mean δ13C (‰) (VPDB); SD	δ13C (‰) (VPDB) Range	Mean δ15N (‰) (AIR); SD	δ15N (‰) (AIR) Range
Early (n = 14)	4400–800 B.C.E.	−17.3 ± 0.8	−18.5 – 16.2	+12.8 ± 1.0	+11.0 – +14.6
Early Iron (n = 7)	800–200 B.C.E.	−16.0 ± 0.8	−16.8 – 14.8	+13.6 ± 1.1	+12.2 – +14.8
Xiongnu (n = 47)	200 B.C.E-250 C.E.	−16.0 ± 1.3	−18.5 – 13.1	+13.2 ± 1.3	+7.9 – +15.5
Mongol (n = 38)	1200–1375 C.E.	−16.5 ± 1.7	−20.4 – 12.4	+12.8 ± 1.7	+6.9 – +16.2
Faunal* (n = 53)	2000 B.C.E. – 200 C.E.	−18.4 ± 1.8	−21.8 – 13.16	+8.1 ± 2.4	+3.5 – +12.6

Table 1. Average bone collagen values for individuals in this study by time period, individual values presented in Supplementary Table 1 (results include additional individuals from previously published articles[20,29,30,44]).

As a consequence, it is perhaps unsurprising that archaeological, archaeobotanical, and historical records are beginning to strongly hint at the possibility that historical Mongolian empires were not solely reliant on dairy pastoralism, but also featured agriculture, as well as craft specialization, and participation in trade systems that spanned thousands of kilometers[12–14].

Of particular interest in this context has been the growing archaeobotanical evidence from across Central Asia that demonstrates an influx of millet, both broomcorn (*Panicum miliaceum* L.) and foxtail (*Setaria italica* L.), and other domesticated grains in the surrounding steppe lands of Siberia, Kazakhstan, and northwestern China during the second and first millennia B.C.E.[15–24]. There are some archaeobotanical data suggesting the use of crops in Mongolia starting around *c*. 100 B.C.E. – 200 CE[25,26], though these have been dismissed as reflective of trade rather than local production[6,27]. Overall, due to issues of wind deflation and a lack of sampling during excavation, archaeobotanical evidence from Mongolia is severely lacking. Moreover, where present, it is difficult to determine the degree to which an archaeobotanical assemblage represents overall dietary reliance. To date, there have been no systematic, direct analyses of the consumption of domesticated crops among peoples in this region over the past three millennia, leaving the economic basis for some of the world's most famous empires unresolved.

Stable isotope analysis of archaeological human and associated faunal remains has emerged as an increasingly powerful methodology for tracking palaeodietary and subsistence change in Central and East Asia[23,28–31]. The distinction in stable carbon isotope ratios ($\delta^{13}C$) between C_3 plants on the one hand – including crops such as rice (*Oryza sativa* L.), wheat (*Triticum* spp. L.) and barley (*Hordeum vulgare* L.) – and C_4 plants on the other – including millets and maize (*Zea mays* L.) – can be tracked through the bone collagen and bioapatite of humans relying on these resources and/or the animals feeding off of them[32]. While bone collagen $\delta^{13}C$ is primarily derived from the protein portion of the diet, tooth enamel bioapatite $\delta^{13}C$ reflects the whole diet[33]. Stable nitrogen isotope ($\delta^{15}N$) analysis provides additional insights into the trophic level position of an individual, orienting them within the local food chain[34].

It is our aim to discover when Eastern Steppe populations began utilizing cultivated C_4 resources (i.e. millet and millet-based foods). We are especially interested in dietary trends during the Xiongnu and Mongol imperial periods, as there has long been a dominant assumption that these empires wholly depended on dairy pastoralism. We use stable carbon isotope analysis of human tissues to directly test whether, in line with some previously published archaeobotanical and historic evidence, the Xiongnu and Mongol empires in fact relied quite significantly on millet-based agricultural systems. We present $\delta^{13}C$ and $\delta^{15}N$ analysis of human bone collagen and $\delta^{13}C$ and $\delta^{18}O$ analysis of human tooth enamel bioapatite from 137 previously-excavated individuals from across Mongolia dated to between c. 4400 B.C.E. and 1375 C.E. in order to directly assess changing diets through the region's key imperial transitions.

Results

Preservation of samples. We analyzed 80 bone collagen and 108 dental enamel samples from 137 individuals from 60 archaeological sites (Tables 1–4; Fig. 1). Samples were separated into four chronological periods based on relative and absolute dating (Early [Neolithic - Bronze Age], Early Iron, Xiongnu, and Mongol; see Supplementary Table S4 for AMS dates). As there is only a single individual from the Neolithic period (c. 4400–3000 B.C.E.), this sample was combined with Bronze Age individuals dating to prior to 800 B.C.E. (n = 23; collagen n = 14, enamel n = 16) to create a single period labelled as 'Early'. The Iron Age samples were split into two chronological periods, corresponding to the pre-imperial Early Iron Age (*c*. 800–200 B.C.E.) and the Xiongnu (*c*. 200 B.C.E. – 250 C.E.). The Early Iron Age samples include 16 individuals (collagen n = 7, enamel n = 16) from one site. From the subsequent Xiongnu, we analyzed 59 individuals (collagen n = 23, enamel n = 54) from 28 sites. Individuals from the later Mongol Empire (*c*. 1200–1375 C.E.) are grouped together and consist of 28 individuals (collagen n = 28, enamel n = 21) from 19 sites.

All of the human bone collagen samples included in this study had atomic C:N ratios between 3.1 and 3.5 and were thus within the accepted range for good collagen preservation[35] (Supplementary Table S1). The collagen yields of these samples ranged between 6 and 30%, with none falling below 1%, a further check of data quality[35]. Furthermore, the majority of collagen samples have greater than 11% N and greater than 30% C, within acceptable ranges[36]. Each bone collagen sample was run in duplicate, and averages are presented in Supplementary Table S1 along with their standard deviation.

Bone collagen carbon and nitrogen stable isotope results. $\delta^{13}C$ and $\delta^{15}N$ results from human bone collagen are grouped into four chronological periods, as detailed above, for comparative analysis. The pre-Bronze and Bronze Age average values are the closest to those of the average faunal values, although the faunal values

Time Period	Date Range	$\delta^{13}C$ (‰) (VPDB)	$\delta^{13}C$ (‰) (VPDB) range	$\delta^{18}O$ (‰) (VPDB)	$\delta^{18}O$ (‰) (VPDB) range
Early (n = 17)	4400–800 BC	−12.9 ± 0.8	−14.3 – −11.9	−10.6 ± 1.0	−11.9 – −8.1
Early Iron (n = 14)	800–200 BC	−11.0 ± 2.1	−13.3 – −5.7	−11.0 ± 0.9	−12.1 – −9.5
Xiongnu (n = 56)	200 BC-250 AD	−11.2 ± 2.3	−14.9 – −3.1	−10.5 ± 1.7	−15.2 – −7.2
Mongol (n = 21)	1200–1375 AD	−11.3 ± 1.9	−15.1 – −6.8	−10.8 ± 2.0	−13.6 – −6.5

Table 2. Average tooth enamel bioapatite values by time period.

have a higher standard deviation. The widest range of carbon and nitrogen isotope values were found in the Xiongnu and Mongol Period populations. For all of the individual $\delta^{13}C$ and $\delta^{15}N$ values from each time period see Supplementary Table 1.

Dental enamel carbon stable isotope results. The data from $\delta^{13}C$ values of human enamel bioapatite are divided into the same chronological periods as the bone collagen data. The Xiongnu population had the largest range of stable carbon isotope values, followed by the Mongol period and Early Iron Age (Table 2). The pre-Bronze/Bronze period had the lowest range of stable carbon isotope values when compared to the later populations. For all of the individual $\delta^{13}C$ and $\delta^{18}O$ values from each time period see Supplementary Table 2 (Samples with both collagen and enamel Supplementary Table 3).

Environmental differences. As stable carbon and nitrogen isotope values may vary in different environments (i.e. temperature and aridity), to adequately assess human $\delta^{13}C$ and $\delta^{15}N$ values from normal steppe (>200 mL of annual precipitation) and dry (<200 mL of annual precipitation) regions, we also determined the average values for each environmental type (Table 3). In these tables we have separated the previously published faunal stable carbon and nitrogen isotope values into the "steppe" or "dry" regions according to modern annual rainfall[37,38].

Statistical tests. Boxplots of our results can be found in Fig. 2A–C, and statistical comparisons between the time periods can be found in Supplementary Table 5 ($\delta^{13}C$ bone collagen), Supplementary Table 6 ($\delta^{13}C$ enamel bioapatite), and Supplementary Table 7 ($\delta^{15}N$ bone collagen). For the bone collagen data, both the Xiongnu and the Mongol average $\delta^{13}C$ values were significantly higher than those of Early individuals ($p < 0.05$). The same trend was seen for tooth enamel $\delta^{13}C$, with Early Iron, Xiongnu, and Mongol samples having $\delta^{13}C$ significantly higher than that of the Early group (for the overall $p < 0.05$, and the specific pairwise comparisons are available in Supplementary Table 6). There was no significant difference between average dental enamel values for the Early Iron, Xiongnu, and Mongol periods $\delta^{13}C$ ($p > 0.05$). Bronze Age $\delta^{15}N$ values were also significantly higher ($p < 0.05$) than those of the Early Iron, Xiongnu, and Mongol periods (Supplementary Table 7).

Isotopic temporal trends in Mongolia and environmental impacts. Higher $\delta^{13}C$ values in individuals from the Early Iron Age, Xiongnu, and Mongol periods could be the product of the increased direct consumption of C_4 crops or wild plants or animals consuming C_4 plants. It should also be understood that both Mongolians and foreign travellers would have been moving within and outside of the imperial borders, and dietary intake likely varied greatly in different regions. In areas with environmentally-linked variation in wild C_4 and C_3 plant distributions, such as Mongolia, it is important to rule out a climatically driven change (see Supporting Information Text 1). Modern plant samples from Mongolia have yielded $\delta^{13}C$ values ranging from −28.3 to −23.4‰ for C_3 photosynthetic pathways and an average $\delta^{13}C$ of −14.7‰ for plants following the C_4 photosynthetic pathway[39]. Notably, wild C_4 plants make up a much smaller proportion of Mongolian and other Central Asian environments than C_3 plants[40,41]. Overall, contemporary studies suggest that leaf $\delta^{13}C$ values decrease with increasing mean annual precipitation[42], both as a product of reduced C_4 plants in wetter landscapes and aridity-driven changes in $\delta^{13}C$ among C_3 plants (see Supplemental Text S1).

While C_4 plants make up a relatively limited portion of the biotic community today, we established local isotopic baselines for Mongolia in the past using archaeological fauna in order to determine if shifts in $\delta^{13}C$ values through time are the product of environmental variations or social and economic choices. Isotopic studies of modern and archaeological herd animals have shown differences in $\delta^{13}C$ values between more and less arid regions[43–45], and that there is variation in the availability of C_3 and C_4 plants across the country[46,47]. While there were no fauna associated with the human remains collected for this study, we were able to use previously published faunal stable isotope data from the Minusinsk Basin of Siberia (just north of Mongolia)(MNSK, AD, AM; n = 21)[20,29], the Gobi (BGC; n = 14)[30,48], Gobi-Altai (SBR; n = 5)[30], and north central Mongolia (EG; n = 13)[30] areas to show that regional herbivores generally consumed C_3 plants, with some having higher stable carbon isotope values, indicative of C_4 plant consumption, in the hyper-arid desert regions[30,48].

Statistical tests further support this assessment, with humans having higher $\delta^{13}C$ values than the available fauna in all periods, with the greatest difference occurring in the Xiongnu and Mongol periods ($p \leq 0.005$) (Figs. 2A and 3A). For terrestrial faunal remains (Fig. 3A), there is a significant correlation between $\delta^{15}N$ and $\delta^{13}C$ bone collagen values ($R^2 = 0.64$, p-value <0.001) which is a product of higher levels of aridity leading to a higher availability of C_4 plants in the natural vegetation cover. However, no such correlation is observed in humans (Fig. 3A,B), either between $\delta^{15}N$ and $\delta^{13}C$ bone collagen values ($R^2 = 0.01$, p-value = 0.15) or between $\delta^{15}N$ bone collagen and $\delta^{13}C$ enamel values ($R^2 = 0.05$, p-value = 0.13). Given this, alongside the consistent elevation of human $\delta^{13}C$ values over the available fauna $\delta^{13}C$ values, this indicates that higher $\delta^{13}C$ values in human bone collagen and enamel is a product of direct consumption of non-wild C_4 plants.

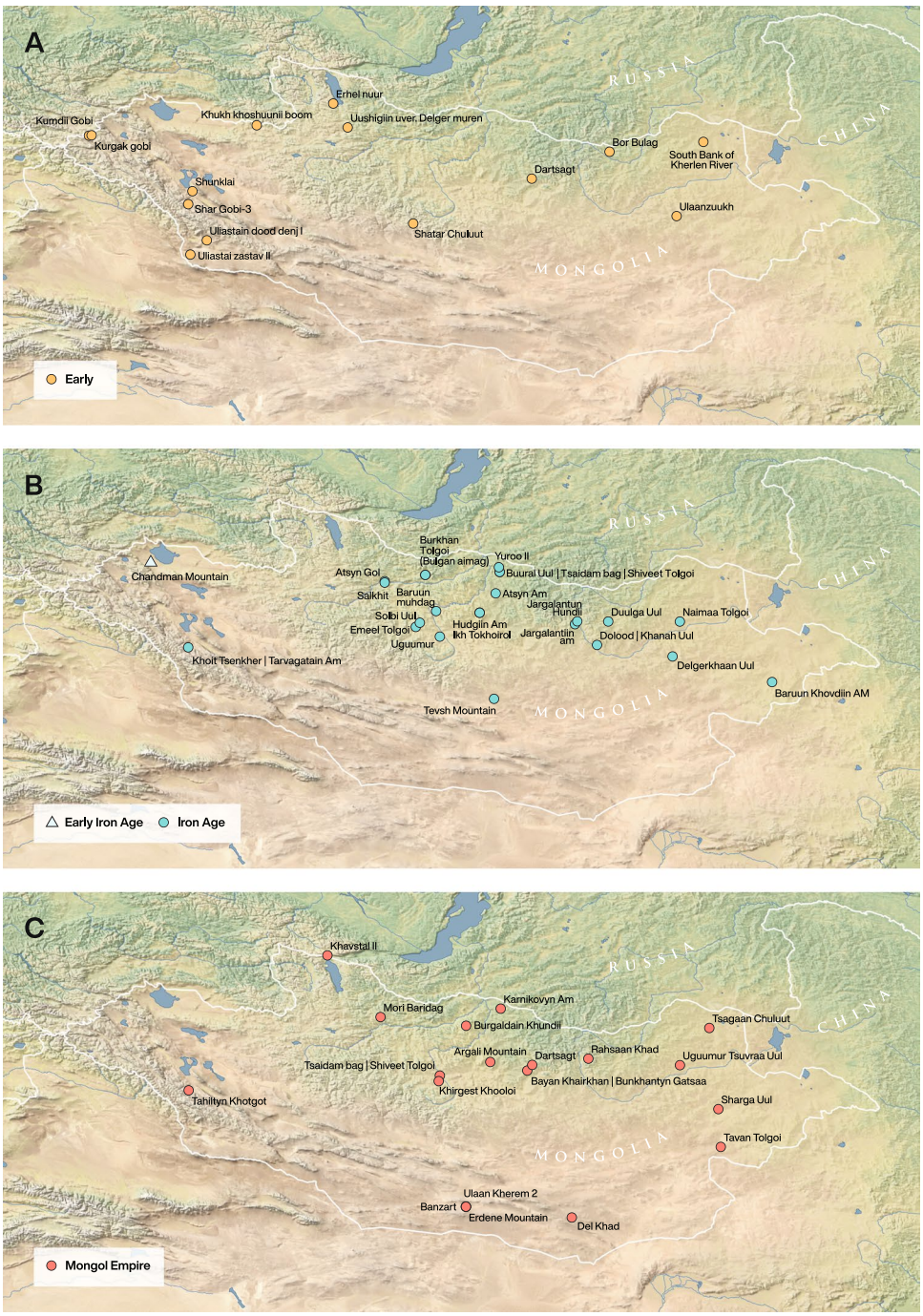

Figure 1. Maps of sites used in this study. These maps were created for this study and were produced using QGIS 3.0[89] https://qgis.org/en/site and using the Natural Early Data maps from https://www.naturalearthdta.com/downloads/ by Shevan Wilkin and Michelle O'Reilly (Graphic Designer for the Max Planck Institute for the Science of Human History, Jena, Germany).

Mean bone collagen $\delta^{13}C$ values for faunal remains from steppe regions are typically C_3 (-19.3 ± 1.3‰), and the stable carbon isotopic offset between bone collagen of herbivores and carnivores is *c.* 1‰[49]. Thus, human bone collagen steppe samples dating to the Early period (prior to 800 B.C.E.) do not show $\delta^{13}C$ values indicative of a millet dietary contribution (-18.3 ± 0.1‰). This same offset applies to human bone collagen samples from dry regions from all periods since these are elevated by up to 1‰ (Early -17.2 ± 0.7‰; Early Iron $= -16.2 \pm 0.9$‰; Xiongnu -16.1 ± 1.1‰; Mongol -16.1 ± 1.4‰) when compared to the bone collagen mean for faunal samples from dry regions (-17.2 ± 1.5‰). However, for later time periods in steppe regions average human bone collagen $\delta^{13}C$ values are elevated by c. 3‰ (Early Iron -16.3 ± 0.7‰; Xiongnu -16.1 ± 1.2‰; Mongol -16.8 ± 2.2‰) when compared to faunal values. Thus, the higher (*c.* 2‰) human bone collagen $\delta^{13}C$ values observed for later periods in steppe regions when compared to the early period is indicative of a temporal increase in millet-based

Time Period	Steppe	Mean $\delta^{13}C$ (‰) (VPDB); SD	Mean $\delta^{15}N$ (‰) (AIR); SD	Dry	Mean $\delta^{13}C$ (‰) (VPDB); SD	Mean $\delta^{15}N$ (‰) (AIR); SD
Early	Steppe (n = 2)	−18.2 ± 0.1	+11.6 ± 0.8	Dry (n = 11)	−17.2 ± 0.7	+13.1 ± 0.8
Early Iron	Steppe (n = 6)	−16.3 ± 0.7	+13.9 ± 1.0	Dry (n = 8)	−16.2 ± 0.9	+13.5 ± 1.1
Xiongnu	Steppe (n = 17)	−16.1 ± 1.2	+12.2 ± 1.1	Dry (n = 30)	−16.1 ± 1.1	+13.7 ± 0.8
Mongol	Steppe (n = 10)	−16.8 ± 2.2	+11.6 ± 2.0	Dry (n = 25)	−16.1 ± 1.4	+13.2 ± 1.3
Faunal	Steppe (n = 34)	−19.3 ± 1.3	+6.8 ± 1.9	Dry (n = 19)	−17.2 ± 1.5	+9.1 ± 2.3

Table 3. Average human and faunal bone collagen $\delta^{13}C$ and $\delta^{15}N$ values between the steppe (>250 mL annual precipitation) and dry (<250 mL annual precipitation) regions.

Time Period	Steppe	Mean $\delta^{13}C$ (‰) (VPDB)	$\delta^{18}O$ (‰) Average	Dry	Mean $\delta^{13}C$ (‰) (VPDB)	$\delta^{18}O$ (‰) Average
Early	Steppe (n = 3)	−14.1 ± 0.2	−11.1 ± 0.3	Dry (n = 4)	−13.0 ± 0.5	−10.4 ± 1.6
Early Iron	Steppe (n = 15)	−11.1 ± 2.0	−10.1 ± 3.6	Dry (n = 0)	n.d.	n.d.
Xiongnu	Steppe (n = 29)	−10.7 ± 2.2	−10.6 ± 1.5	Dry (n = 4)	−12.3 ± 1.0	−10.5 ± 1.5
Mongol	Steppe (n = 5)	−10.6 ± 2.3	−12.6 ± 1.0	Dry (n = 5)	−11.7 ± 1.1	−9.6 ± 2.6

Table 4. Average human dental enamel $\delta^{13}C$ and $\delta^{18}O$ values between the steppe (>250 mL annual precipitation) and dry (<250 mL annual precipitation) regions.

food consumption. Furthermore, given that bone collagen reflects primarily consumed protein, and that millet has a poor protein content, the dietary caloric contribution from millet was likely much higher than its protein contribution[50]. This is corroborated by human enamel $\delta^{13}C$ values given that this isotopic proxy reflects the carbon dietary mix[50]. Steppe human enamel samples for the later periods show mean $\delta^{13}C$ values higher by *c.* 3.5‰ when compared to the Early period. For dry areas, mean human enamel samples $\delta^{13}C$ values are higher by *c.* 1‰ when compared to the Early period, which indicates a temporal increase in millet-based food consumption although considerably smaller than that observed in the steppe regions as shown also in the model estimates for millet caloric contributions (Fig. 4).

Further evidence for C_4 plant consumption is offered by the distribution of isotopic values. For faunal remains there is a positive significant correlation ($R^2 = 0.67$, p-value <0.05, correlation coefficient =1.126) between $\delta^{15}N$ and $\delta^{13}C$ bone collagen values which is expected given that an increase in aridity leads to a higher availability of C_4 plants in the vegetation cover. A similar correlation, albeit with isotopic offsets, would be expected if humans relied exclusively on animal products. However, no clear environmentally-driven correlation is observed for the human groups. There is no significant correlation for the Early ($R^2 = 0.65$, p-value <0.08, correlation coefficient = 0.65) and Xiongnu ($R^2 = 0.0$, p-value <0.46, correlation coefficient = 0.11) periods, and although the correlation is significant for the Mongol period ($R^2 = 0.13$, p-value <0.02, correlation coefficient = 0.39), it only explains 39% of the variability. For similar $\delta^{15}N$ bone collagen values across the human individuals, there are wide ranges in $\delta^{13}C$ collagen values. Whereas during the Xiongnu period one can observe a significant negative correlation ($R^2 = 0.0$, p-value <0.46, correlation coefficient = 0.11), which implies the contribution from a food source with higher $\delta^{13}C$ values but lower $\delta^{15}N$ values when compared to animal food sources. These isotopic relationships are indicative of varying individual intake of a food with elevated $\delta^{13}C$ values, such as millet, and having relatively uniform $\delta^{15}N$ and $\delta^{13}C$ values across regions with varying levels of aridity.

Bayesian spatial modelling of C_4 plant caloric consumption. To further confirm that the increased $\delta^{13}C$ values in human bone collagen and tooth enamel through time is a product of the consumption of crops rather than changing availabilities of baseline C_4/C_3 plant ratios or the availability of samples in different local environments, we developed a Bayesian model to produce a C_4 dietscape, representing estimates of spatial distribution of C_4 plants based on per capita caloric consumption (See SI for detailed discussion). Stable carbon isotope data of dental enamel was used, and individuals were separated into two periods, Early (Neolithic - Bronze Age) or Late (Xiongnu, and Mongol). The results for the two models show that during the Early period C_4 caloric contributions were very low across Mongolia, likely including consumption of local plants and livestock consuming natural vegetation, with mean estimates varying between *c.* 2.5 and 5.0% of calories (interpolation 1-sigma uncertainty up to 0.5% calories) (Fig. 5A,B). During the later periods, the variability in millet-based food consumption increases considerably as shown by the range in the mean estimate (between 3 and 26% of per capita millet calories) and in the 1-sigma interpolation uncertainty for each location (between *c.* 3 and 6% of per capita millet calories) (Fig. 5C,D). The C_4 plant dietscape for the late period also shows that millet consumption is concentrated in central northern Mongolia (reaching the highest mean value [26% per capita millet calories]), an area where environmental increase of carbon values would not be expected naturally (Figs. 4 and 5).

Discussion

Isotopic indicators of diet through time in mongolia. Our results clearly demonstrate an increase of human consumption of C_4 plants during the imperial periods in ancient and historic Mongolia (Figs. 2, 3, and 5). While high $\delta^{15}N$ values in human bone collagen relative to the faunal data (Fig. 2A and 3A) supports evidence for human reliance on dairy and meat products throughout the periods under study, the change in C_4 plant

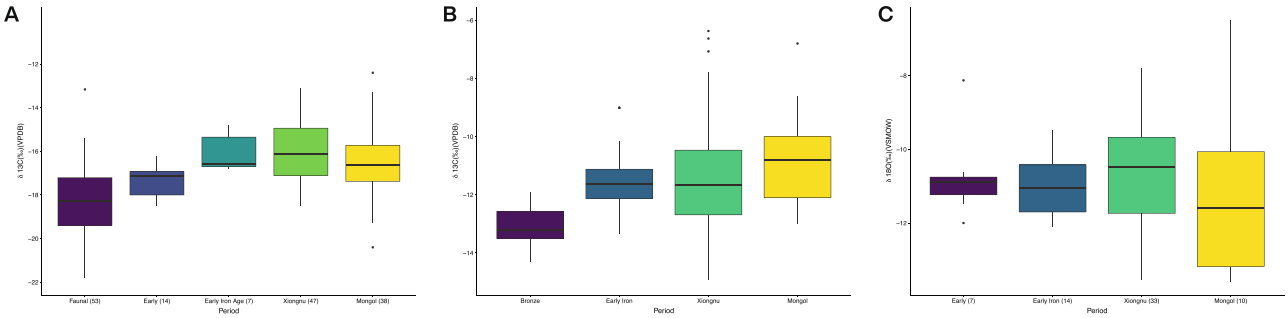

Figure 2. Boxplots showing the range of carbon values for all individuals from each period. Outliers are shown as individual data points. (**A**) Comparison of the bone collagen carbon values for humans and fauna. Faunal data derives from previously published data[20,29,30,44], and all human data is from this study. (**B**) Difference in human enamel values between Early, Early Iron, Xiongnu, and Mongol periods. The Early Iron, Xiongnu, and Mongol period average values are significantly higher than the Early period average. (**C**) Boxplots showing the range of oxygen values from enamel samples. There are no significant differences between any of the time periods.

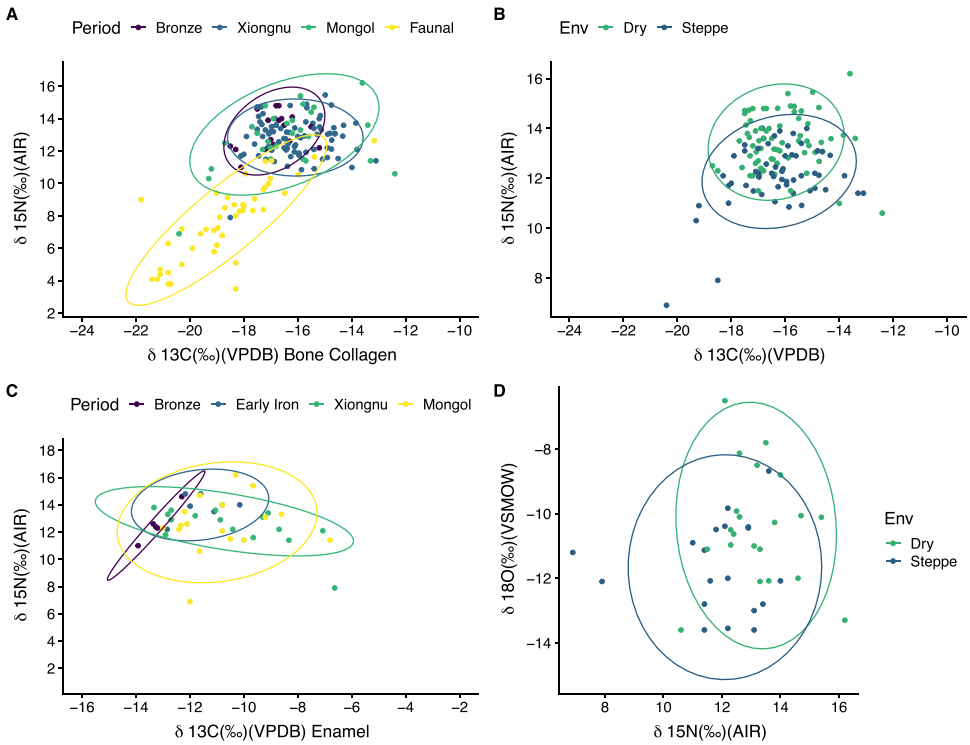

Figure 3. Carbon and nitrogen values from bone collagen with ellipses showing ranges at 95% confidence. (**A**) Individuals included in this study as well as humans and faunal values from previously published data[31] (**B**) Humans included in this study showing the variation between those in the "Dry" and "Steppe" zones. "Dry" sites have less than 250 mm of annual precipitation, "Steppe" sites have over 250 mm of precipitation per year. (**C**) $\delta^{15}N$ from bone collagen versus $\delta^{13}C$ values from dental enamel demonstrating the shift from primarily C_3 reliant diets in the Early period to a wider range of carbon and nitrogen values, indicating an increase in the diversity of diets in the later three periods. (**D**) $\delta^{18}O$ from dental enamel versus $\delta^{15}N$ from bone collagen showing the values in "Dry" and "Steppe" areas.

consumption represents the major dietary shift within this timeframe. The significant decrease in $\delta^{15}N$ values in the later periods, in comparison to the Bronze Age, further supports this point, potentially indicating reduced consumption of meat and milk and increased consumption of grains. Moreover, comparisons with faunal datasets and environmental background data allows us to confidently state that this shift is a consequence of increasing rates of consumption of C_4 resources. Stable carbon isotope values from individuals before the Early Iron Age show little indication of C_4 plant consumption beyond the local natural baseline identified through the fauna from similar environments. Individuals from the cemetery of Chandman Mountain (c. 900–400 B.C.E.)

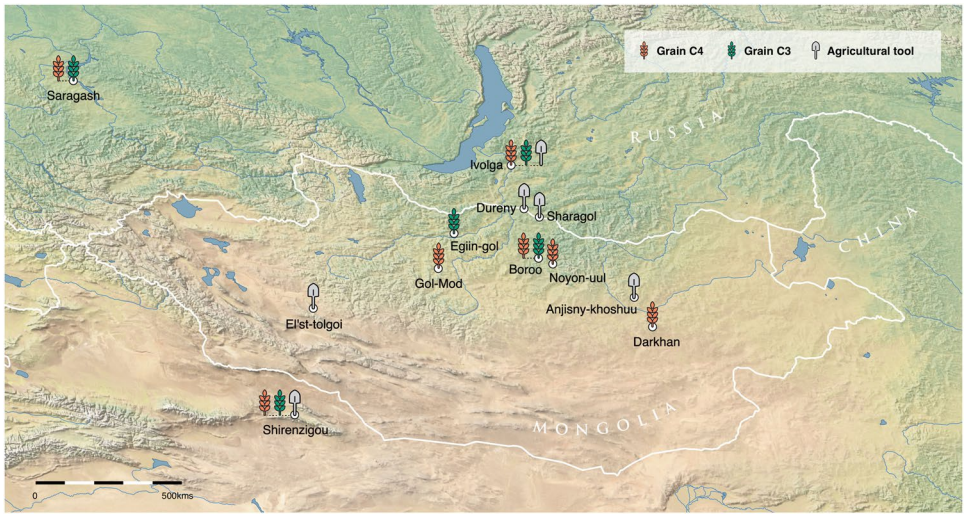

Figure 4. Sites in and around Mongolia with archaeological or archaeobotanical evidence for C_3 (wheat and barley) and C_4 (broomcorn and foxtail millet) grain cultivation during the Iron Age. This map was newly created for this study and produced using QGIS 3.0[89] https://qgis.org/en/site and using the Natural Early Data maps from https://www.naturalearthdta.com/downloads/ by Shevan Wilkin, Bryan K. Miller, and Michelle O'Reilly (Graphic Designer for the Max Planck Institute for the Science of Human History, Jena, Germany).

analyzed in this study show the first visible evidence of C_4 plant consumption as part of a mixed agro-pastoral diet. However, this particular site in northwest Mongolia is more a part of the Minusinsk Basin region of southern Siberia, an area where millet consumption was common by the Late Bronze Age, than the rest of Mongolia. Previously published contemporaneous human and faunal isotope analyses within this region are indicative of animals consuming primarily C_3 plants[20], with the human population postulated as having a diet additionally composed of C_4 plants in concert with meat and milk.

In the rest of Mongolia during the tenure of the Xiongnu and Mongol empires human stable carbon values became more varied with increasing numbers of individuals displaying bone collagen and tooth enamel $\delta^{13}C$ values suggestive of moderate to high C_4 plant consumption, with the number of individuals with such values reaching their peak during these imperial periods. We also observe the largest range and diversity of $\delta^{13}C$ and $\delta^{15}N$ values during the imperial periods. This is likely due to diverse subsistence strategies being pursued across each empire, reflecting different environmental zones and levels of imperial support. This is result of the extensive range of each empire, and includes the knowledge that not everyone that died in Mongolia would have been "Mongolian", but these individuals likely lived and died within the empires. Since the majority of the individuals analyzed were excavated from elite imperial tombs, and human remains representing other sectors of society are lacking at present, attributing all outliers to non-local outsiders would be to dismiss the agency of Mongolian populations and provide something of a 'colonial' narrative.

Individual bone collagen and tooth enamel $\delta^{13}C$ values for the Xiongnu and Mongol empires range between those indicative of a pure C_3 diet to those that suggest heavy C_4 plant consumption. Interestingly, during this period, a few individuals had $\delta^{13}C$ values lower than those of the Early period which, alongside lower $\delta^{15}N$ values, indicates a staple intake of C_3 plants, likely crops such as wheat and barley. Historical and archaeobotanical sources suggest that cereal crops were commonly cultivated or obtained through trade during the Mongol period[13,51–56]. In addition to grains, carbonized fruit and nut remains have been recovered from sediments at the Mongol capital of Kharakhorum (also used during the Mongol rule in the Yuan Dynasty) showing the diversity of imported plants through the presence of rice (*Oryza sativa* L.), over a dozen cultivated fruits, including grapes (*Vitis vinifera* L.), figs (*Ficus carica* L.), and jujube (*Ziziphus jujube* Mill.), as well as vegetable and oil-seed crops. There are also remains of spices – notably a few, such as black pepper (*Piper nigrum* L.) and caraway (*Carum carvi I.*), that were imported along the trade routes with South Asia, and would have involved transport across distances of up to 2000 kilometers[12].

The resulting bone collagen $\delta^{13}C$ and $\delta^{15}N$ values have been plotted to show this increase of dietary diversity over time (Fig. 2A). From our data, alongside the growing corpus of biomolecular, archaeological, and historical data, it is evident that the Xiongnu and Mongol Empires had complex imperial structures that facilitated increasingly diverse subsistence economies. The combination of crop cultivation in tandem with dairy pastoralism would have allowed these empires to sustain a diverse economic surplus that defended against livestock depletion from harsh winters, crop loss, or volatile political episodes. Diverse dietary values likely also reflect an increasingly cosmopolitan society in which dietary heterogeneity within populations increased with growing migration, trade and interaction, and the emergence of increasingly elaborated elite statuses. The diversity could also reflect temporal political shifts within the time-span covered by our sampling groups, with trade routes to Karakorum decreasing in volume during the Mongol Period after the switch of the capital in 1260 and ending with the end of the Yuan Dynasty in 1368[12,57], for example.

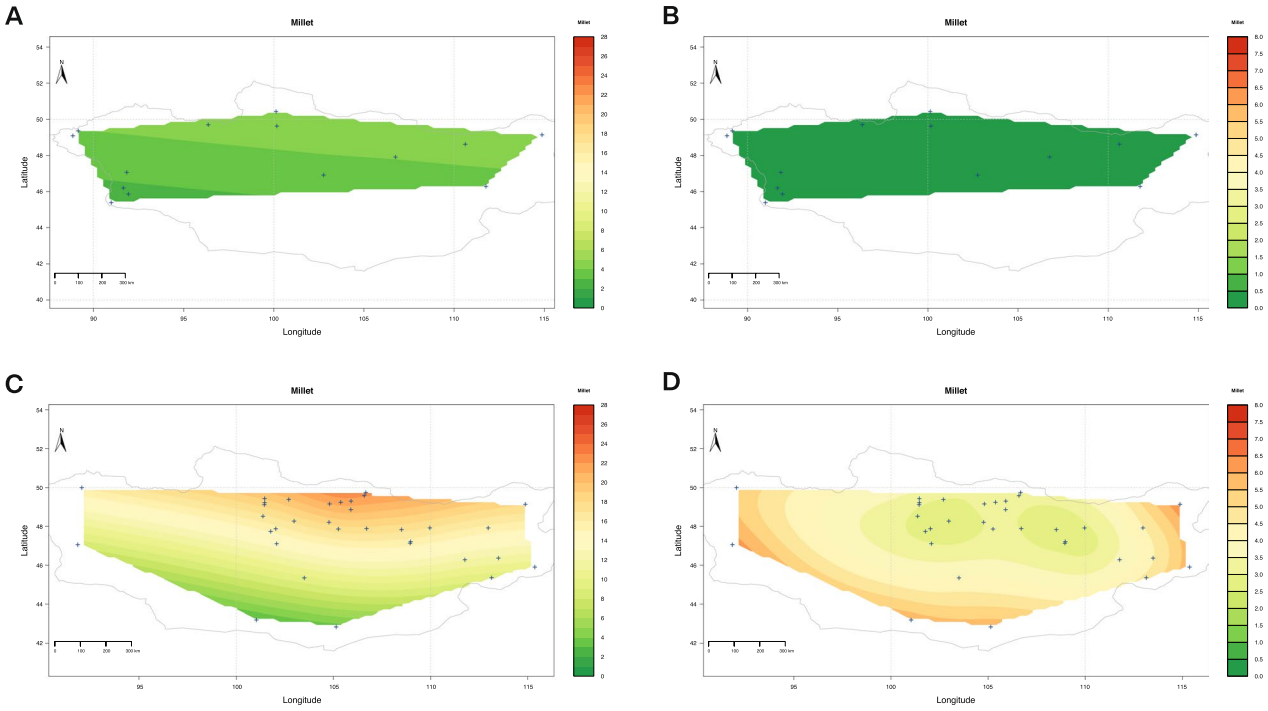

Figure 5. Dietscape representing average millet caloric consumption per capita. (**A**) Average millet caloric consumption for the Early period estimated through Bayesian modelling of dental bioapatite carbon stable isotope values. (**B**) Standard error of the mean for millet caloric consumption average during the Early period. (**C**) Average millet caloric consumption for the Late period estimated through Bayesian modelling of dental bioapatite carbon stable isotope values. (**D**) Standard error of the mean for millet caloric consumption average during the Late period.

Mongolian empires in context. Historical and ethnographic research indicates the importance of pastoralism on the historic and proto-historic eastern Eurasian Steppe. Recent proteomic research has demonstrated the clear importance of dairy-based pastoralism to Mongolian dietary practices from at least c. 1500 B.C.E.[58]. Nevertheless, our data clearly highlight that pastoral lifestyles did not preclude the inclusion, and later intensification, of crop use. Millet's suitability to arid environments combined with its short growing period is compatible with the often peripatetic, mobile lifestyles of pastoralists[22,26]. Indeed, during the Xiongnu and Mongol empires, we see clear evidence for human dietary reliance on millet in a significant proportion of individuals. Although some scholars contend that all grains were either extorted or imported from China and other exterior polities[6], we argue that our data, alongside existing archaeobotanical and archaeological findings[25,59,60], provide clear evidence for imperial reliance on locally grown crops in the Xiongnu and Mongol heartlands, as well as the coordination of diverse economic connections and exchanges[12]. These discoveries bolster the notion of an economically diverse population across much of Mongolian history[14,61,62].

Agricultural tools for plowing, hoeing, and grinding have been uncovered from permanent Xiongnu settlements in Mongolia, implying local plant cultivation and processing[63], and charred remains of millet, barley, and wheat grains have been recovered through flotation at pit-house villages at Boroo[64] and ephemeral campsites[61]. Studies in the Egiin Gol valley and at the large site complex of Ivolga have illustrated the presence of long-season cereal crops (wheat and barley) in the Iron Age, which represent more labor investment in farming practices than millets[61,63]. At Ivolga, this occurs alongside evidence of ploughshares at permanent settlements[63], as well as written accounts of crops suitable for the northern steppe being managed by imperial Xiongnu administrators, such as the 'Lord of Millet Distribution', referred to in the 1st century C.E. Chinese accounts[54]. Millet grains, still articulated in their chaff, have been found within the graves of Xiongnu rulers at Gol Mod and Noyon Uul[27] as well as of local elites throughout the steppe[60]. There are also uncharred grains found within Xiongnu pit-house villages, all of which were unprocessed (i.e. with palea and lemma) and thus most likely not transported long distances[60] instead representing local production and consumption (Fig. 4).

Scholars working in Mongolia have extensively discussed the formation of hierarchical political systems and greater concentrations of population densities in the absence of farming, often describing imperial systems in Central Eurasia as unique due to their economic basis[65–69]. In other parts of Asia, farming is linked to demographic expansion and the congregation of greater population densities[70]. Notably, millet farming is linked to urbanization[71,72] and imperial formation[73] in East Asia. Boserupian economics suggest that increased investment in farming, along with a diversified economy and higher levels of cultural exchange, often lead to a demographic transition[74–76]. The data presented in this paper suggests that, while Mongolian empires have often been seen as outliers in global comparisons of imperial structures, they were in fact, like many others around the world, highly reliant on economic diversification, local adaptations to a diversity of environments, and the creation of reliable and stable subsistence resources and economic surpluses[8–10].

Mongolian empires have traditionally conjured up exotic ideas of mobile pastoral specialists who roamed the Asian steppes attacking more sedentary communities[1–4]. While prominent in the public sphere, such preconceptions have also directed the type of questions academics have asked. For example, comparative analysis of Mongolian empires with others around the world has been limited, with 'Steppe Empires' often portrayed as deficient or somehow doomed to failure in the absence of reliable crop-based surplus[6,77]. As in other parts of Central Asia, where occupation sites have been hard to come by[78,79], simplistic projections of ethnographic and ethnohistoric datasets into the past have been common in Mongolian archaeology. We hope to have demonstrated how multidisciplinary approaches, built on datasets from different parts of Mongolian imperial networks, can begin to provide novel insights into their economic systems and, perhaps most importantly, their geographic and temporal variability. While there is no doubt that the Xiongnu and Mongol empires were unique, they were also built upon many of the same tenants of economic diversity, stability, and reliability that have characterized imperial structures throughout prehistory and history, demonstrating the importance of a core set of underlying variables in both enabling and driving the formation of empires.

Methods and Materials

Sites and materials analyzed. All bone and tooth samples included in this study were collected from the National University of Mongolia's Department of Archaeology during the winter of 2016. Bone collagen was analyzed for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes, and the carbonate of dental enamel bioapatite was measured for carbon stable isotopes ($\delta^{13}C$), with some individuals analyzed for both bone collagen and bioapatite (see Supplementary Table 3). Time periods for samples ranged from the mid-fifth millennium B.C.E. to the Mongol Empire, as dated by AMS radiocarbon methods where possible (see below).

Samples of bones and teeth were collected from archaeological sites across the country of Mongolia, through varying environmental and topographical zones. Where possible, we collected a tooth and long bone fragment from individuals from each time period. While we aimed to assemble an equal number of samples from all time periods, the collection was dominated by individuals from the imperial periods, resulting in fewer individuals prior to the Iron Age. Bone collagen was preferably extracted from rib bones, but occasionally other bone fragments were employed (clavicle, femur, crania). $\delta^{13}C$ and $\delta^{15}N$ stable isotope measurements of human bone collagen inform primarily on protein source[80], and the bones sampled (i.e. ribs) represent a period of diet of approximately the last 20 years of life[80].

By contrast, tooth enamel $\delta^{13}C$ values are indicative of the whole dietary carbon (carbon mix of protein, lipids, and carbohydrates) consumed during enamel formation[33,49]. First molars mineralize before an individual is 3 years old, second molars are fully formed around age 8, and third molars, if present, are completely mineralized between the age of 7 and 16[81]. To avoid tooth samples that might show a breastfeeding isotopic signal contribution in older children and adolescents we preferentially selected second and third molars. First molars were chosen only when both the M2 and M3 were unavailable.

Stable isotope analysis methods. *Bone collagen.* We selected ribs for bone collagen analysis as representative of the last *c.* 20 years of life[80]. Collagen was extracted from each rib sample following standard procedures[35]. Approximately 1 gram of pre-cleaned bone was demineralized in 10 ml aliquots of 0.5 M HCL at 4 °C, with changes of acid until CO_2 stopped evolving. The residue was then rinsed three times in deionized water before being gelatinized in pH 3 HCl at 75 °C for 48 hours. The resulting solution was filtered, with the supernatant then being lyophilized over a period of 24 hours.

After calculating the collagen yield, all purified collagen samples (~1 mg) were located in tin capsules to be analyzed in duplicate at the Department of Archaeology, Max Planck Institute for the Science of Human History by the elemental analyzer/continuous flow isotope ratio mass spectrometry (EA-IRMS) using a ThermoFisher Elemental Analyzer coupled to a ThermoFisher Delta V Advantage Mass Spectrometer via a ConFloIV system. $\delta^{13}C$ and $\delta^{15}N$ values were compared and calibrated against International Standards (USGS40 ($\delta^{13}C_{raw} = -26.4 \pm 0.1‰$, $\delta^{13}C_{true} = -26.4 \pm 0.0‰$, $\delta^{15}N_{raw} = -4.4 \pm 0.1‰$, $\delta^{15}N_{true} = -4.5 \pm 0.2‰$), IAEA N2 ($\delta^{15}N_{raw} = +20.2 \pm 0.1‰$, $\delta^{15}N_{true} = +20.3 \pm 0.2‰$), IAEA C6 ($\delta^{13}C_{raw} = -10.9 \pm 0.1‰$, $^{13}C_{true} = -10.8 \pm 0.0‰$) Replicate analysis of an in-house fish gelatin standard suggests that machine measurement error is *c.* 0.1‰ for $\delta^{13}C$ and 0.3‰ for $\delta^{15}N$.

Tooth enamel. Teeth or tooth fragments were cleaned using air-abrasion to remove any adhering external material. 8 mg of enamel powder was obtained using gentle abrasion with a diamond-tipped drill along the full length of the buccal surface or fragment in order to maximize the period of formation represented by the resulting isotopic analysis for bulk samples. Enamel powder was pre-treated using a protocol to remove any organic or secondary carbonate contaminates[21]. This consisted of a series of washes in 1.5% sodium hypochlorite for 60 minutes, followed by three rinses in purified H_2O and centrifuging, before 0.1 M acetic acid was added for 10 minutes, followed by another three rinses in purified H_2O (as per[35]).

Following reaction with 100% phosphoric acid, gases evolved from the samples were analyzed to stable carbon and oxygen isotopic composition using a Thermo Gas Bench II connected to a Thermo Delta V Advantage Mass Spectrometer at the Max Planck Institute for the Science of Human History, Jena (MPI-SHH). Carbon and oxygen isotope values were compared against an International Atomic Energy Agency (NBS 19) and in-house standard (MERCK). Replicate analysis of internal bovid enamel standards suggests that machine measurement error is *c.* ± 0.2‰ for $\delta^{13}C$ and ± 0.2‰. Using a Thermo Gas Bench 2 in tandem with a Thermo Delta V Advantage Mass Spectrometer at MPI-SHH, gases produced from a reaction with 100% phosphoric acid were analyzed for stable carbon and oxygen isotopic composition. We compared the resulting values against International Standards (IAEA-603 ($\delta^{13}C = 2.5$; $\delta^{18}O = -2.4$); IAEA-CO-8 ($\delta^{13}C = -5.8$; $\delta^{18}O = -22.7$); USGS44 ($\delta^{13}C = -42.2$)); as well as an in-house

standard of (MERCK ($\delta^{13}C = -41.3$; $\delta^{18}O = -14.4$)). The data from these standards suggest that the machine measurement error is *c.* $\pm$ 0.1‰ for $\delta^{13}C$ and $\pm$ 0.2‰ for $\delta^{18}O$. We increased the precision of our analyzed samples by measuring repeats of extracts using a tooth enamel bovid standard (n = 20, $\pm$ 0.2‰ for $\delta^{13}C$ and $\pm$ 0.3‰).

Statistical tests. To determine whether the differences in human $\delta^{13}C$ between each period were significant, we performed a Wilcoxon rank sum test, with multiple test correction using the Benjamini-Hochberg procedure. All tests were performed using the free R statistical software[82].

Bayesian dietary modelling. Caloric estimates of millet intakes were obtained using the Bayesian mixing model FRUITS having as input data individual tooth enamel $\delta^{13}C$ values and local food isotopic values adjusted for spatial variations due to varying environmental conditions[80]. To achieve the latter, we grouped site locations into the categories of "steppe" and "dry" depending on modern day annual precipitation. Steppe sites have a range from 250–350 mm in precipitation per annum and arid sites have below 250 mm of yearly rainfall. It was assumed that the enamel $\delta^{13}C$ signal is defined by the dietary carbon mix[50]. To extrapolate the spatial distribution of per capita millet caloric intakes (dietscape) a Bayesian additive mixed model with error-in variables[83–85] available as an online app via the Pandora & IsoMemo initiatives was employed[86]. Dietscapes were generated for two main periods corresponding to a temporal divide defined by the intensification of millet consumption as observed from the interpretation of raw isotopic data, into Early (Bronze Age) and Late (combining the Early Iron Age; Xiongnu; Mongol periods). Modelling at a higher chronological resolution was not possible given a lack of data for shorter time periods. Further details on dietscape modelling are available in Supplementary Text S3.

Radiocarbon and archaeologically classified dates. AMS radiocarbon dates were conducted at the Oxford Radiocarbon Accelerator Unit (ORAU), Oxford, England, UK (n = 14; bone collagen and dentine)[87] and at the University of Groningen, Faculty of Science and Engineering, Groningen, The Netherlands (n = 25; bone collagen and dentine)[88]. All pre-Xiongnu samples and 30% of the Xiongnu samples were radiocarbon dated to solidify the dating of individuals from early time periods. Most Xiongnu and Mongol samples were separated into periods based on archaeological materials and burial styles as assessed by excavators and curators from the National University of Mongolia. See Supplementary Text S2 for additional details.

Data availability and ethical approval statement. All of the data included in the study have been made available in Tables 1 and 2, the Supplementary Information. Samples analyzed for this study (AT- denoted codes) are currently curated at the Max Planck Institute for the Science of Human History, Jena, Germany. Samples were exported to the Max Planck Institute for the Science of Human History under permission from the Ministry of Culture, Education, Science and Sports (Export number 10/413 (7b/52) which was received on 2nd February, 2017 #A0109258, MN DE 7 643)

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Author contributions

S.W., P.R. and N.B. designed the experiments. S.W., M.B., S.U. and E.M. collected the samples. S.W. and J.Z. completed laboratory analyses. S.W., A.V.M., R.F., R.H., J.Z. and P.R. analyzed the data. S.W., P.R., A.V.M., B.K.M., R.F., W.T., R.N.S., S.U. and E.M. contextualised the data and wrote the article with input from all authors.

Competing interests

The authors declare no competing interests.

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6. Discussion

Together, these three, independent studies illustrate the evolution of subsistence patterns over 6000 years in ancient and imperial Mongolia. This research clearly shows the presence of ruminant dairy consumption from the Early Bronze Age (ca. 3000 BCE) (Manuscript B), which extends the date of the earliest dairying on the eastern steppe over 1500 years earlier than previously established (Manuscript A). Importantly, the earliest evidence for dairy in this study was identified from individuals in Afanasievo burial sites, supporting our hypothesis that dairy was integrated into Mongolia through Early Bronze Age migrations of western steppe herding groups crossing the Altai. As detailed in Manuscripts A and B, our studies demonstrated that while the first dairying practices were introduced by western steppe groups, by *c*. 1300 BCE, dairying had been adopted by local northern populations with very little western steppe ancestry.

The identification of horse milk proteins in the Late Bronze Age (*c.* 1200 BCE), concurrent with equine skeletal evidence for horseback riding, is especially informative as both traditions have persisted for millennia and are still commonly practiced today (Manuscript B). That Bronze Age Mongolian horses are a distinctly different equine species that those from the Eneolithic Botai sites in northern Kazakhstan [114], a site with lipid evidence suggestive of horse dairying [27]. Our data may represent either an independent emergence of horse dairying, or possibly, the first evidence for horse milk consumption. Whichever scenario these data represent, we show that in the Late Bronze Age there was a dramatic shift in how people on the eastern steppe used horses in their subsistence and economic strategies (Manuscript B).

Our third study illuminated dietary differences between the pre-imperial populations and those of the later Xiongnu and Mongol Empires. The individuals in the "early" groups (Eneolithic and Bronze Age) did not display carbon values indicative of moderate- to high-level C_4 consumption, while the imperial individuals had carbon values suggestive of low- to high-input C_4 grain consumption - likely as part of a diversified dietary economy. A significant decrease in $\delta^{15}N$ ratios from the "early" to "late" periods further supports a decrease in the dependence on meat and dairy once the consumption of cultivated grains became more common (Manuscript C).

6.1 *Dairying in ancient Mongolia*

Recent studies combining ancient genomics with archaeological data discuss the migrations of the dairy-focused pastoral Yamnaya populations north of the Caspian Sea at 3000 BCE across the central steppe [23,25,115]. Our data support the introduction of dairy pastoralism into Mongolia by western steppe herders moving across Eurasia and over the Altai Mountains. The earliest individual in our sample set, from an archaeologically-identified Afanasievo site in central Mongolia, is from a clear western steppe mitochondrial haplogroup and contains peptides from a sheep/cattle milk proteins [116]. This early evidence of western steppe style burial mounds in the central Mongolian Khangai Mountains concurrently with Yamnaya populations in the far western steppe [23] indicates that the Afanasievo populations spread further into East Asia than originally thought (Figure 9).

While the first evidence of dairy consumption in Mongolia is found at Afanasievo and Chemurchek Early Bronze Age burial sites (*c.* 3000 - 2500 BCE), Late Bronze Age dairy reliant sites in northern Mongolia have a very different demographic profile. In the first study of this thesis, ancient DNA analysis revealed that while 9 of the 11 calculus samples contained dairy proteins, the associated individuals were of a local ancestral background, rather than from the western steppe [74]. This indicates that although early dairying was likely introduced from the migrating western steppe herders (Manuscript B), the practice was thoroughly adopted by local Mongolian populations over the subsequent 1500 years.

Evidence for the consumption of horse dairy beginning around 1200 BCE is especially important, as this coincides chronologically with the earliest morphological evidence for horse riding in Mongolia [117–119]. The incorporation of horse riding and milking into pastoralist systems resulted in dramatic demographic changes and shifts in occupation site location strategies (Figure 9). Horses offer faster ways to travel, and with the addition of horses into the pastoral economy, herding larger numbers of animals becomes possible [120]. Furthermore, horses can use their hooves to break through the snow cover allowing themselves and other taxa in the herd to access the plant sustenance below, which is especially important over the frigid Mongolian winters. In the Late Bronze Age, our protein data combined with morphological skeletal indications of horse riding [119–121], and evidence of occupation sites in previously unoccupied intermontane areas of the steppe [118] suggest a dramatic shift in the

economic role of horses in Mongolia, as well as changes in the efficiency of pastoral resource systems.

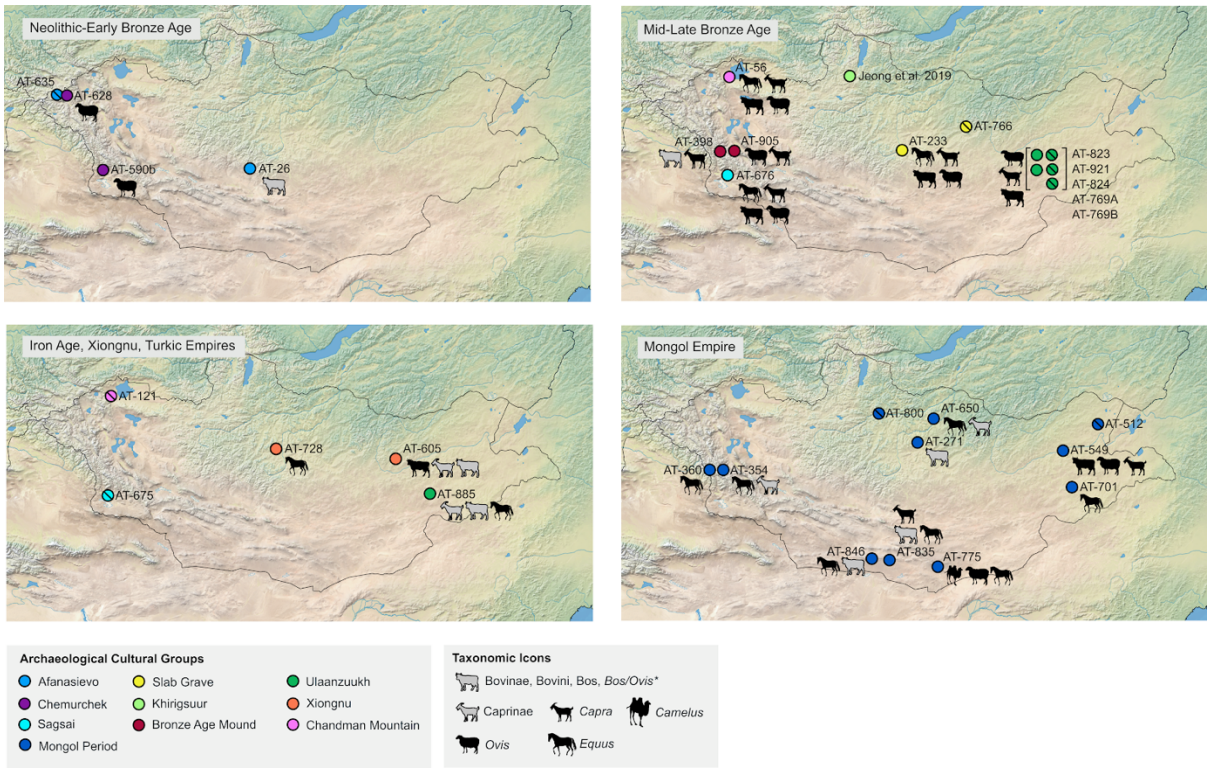

Figure 9. Four panel map of individuals analysed in this study, showing archaeological culture group affiliations, the presence or absence of dairy consumption and taxa consumed. Solid filled circles indicate individuals with a positive identification of dairy consumption. Circles with a line-through indicate no dairy consumption detected. Individual AT-923, associated with Ulaanzuukh, is not directly radiocarbon dated and is not included in this figure. Taxonomic icons only indicate the most specific taxa identified in a phylogenetic branch. The full list of dairy species identified for each individual is listed in Table 1 of Manuscript B.

6.2 *The diversification of diets in the imperial periods*

Our protein study demonstrates that dairy pastoralism continued as a part of subsistence strategies through the Xiongnu and Mongol periods. However, our study into stable carbon isotope analysis of human remains across these time periods establishes a significant increase in millet consumption by the Xiongnu period, demonstrating that the imperial economy was diverse, including both dairy and cultivated grains. As archaeological and botanical studies have identified cereal grains as well as tools for farming and processing we can see that the use of locally grown grains was an important part of eastern steppe diets by the Iron Age.

Through the combination of stable carbon isotope data with established archaeological and historical evidence, we can see the presence of local millet cultivation in concert with other grains acquired through long distance trade. The significant increase in dietary diversity during the imperial periods (Figure 10) demonstrates the innovative ways each empire increased surpluses to combat food shortages, and confirms that these empires relied on complex subsistence systems. The focused, and possibly high-investment cultivation of millet within the empire secure the Xiongnu and Mongol Empires as complex political systems relying on multi-resource agro-pastoral economies. These new data, combined with the established historic and botanical evidence, have overturned the long-standing, popular assumptions of Mongolian empires as primarily dairy pastoralists that derived grains from neighbouring regions.

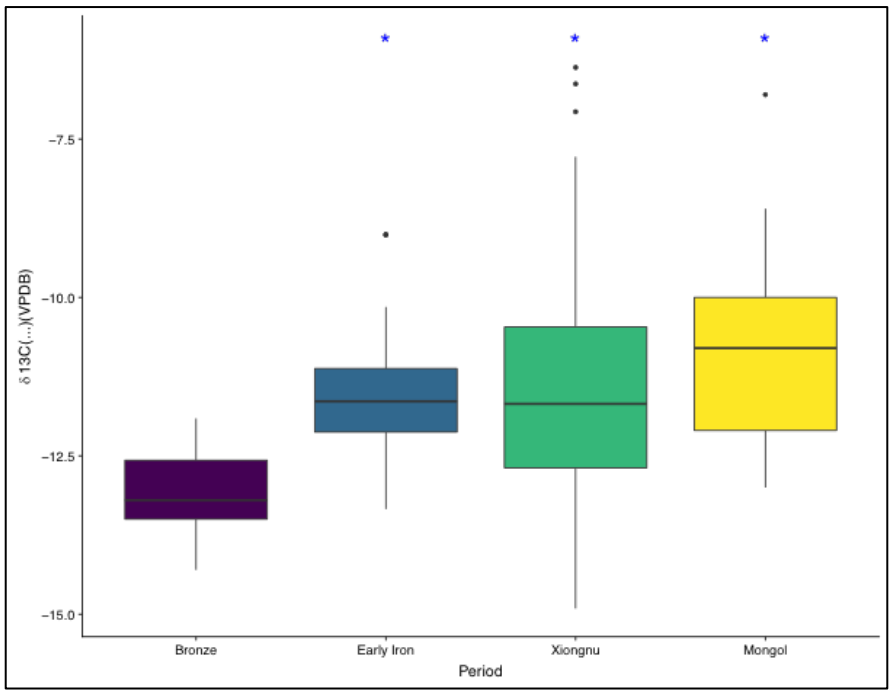

Figure 10. Boxplot showing the range of $\partial^{13}C$ values for all dental enamel samples in each time period. Difference in human enamel values between Bronze, Early Iron, Xiongnu, and Mongol periods. The Early Iron, Xiongnu, and Mongol period average values, noted with * are significantly higher than the Bronze Age average.

6.3 *Future directions*

Archaeological studies, including lipid and protein analyses, have spatially and temporally bookended the origin and extent of the spread of ruminant dairying across the Eurasian steppe [10,11,74]. While it is evident that western steppe populations with pastoral dairy herds migrated eastward from Neolithic Anatolia across the steppe into

Mongolia, the pathways via which these pastoral populations traveled remains unclear. Also unclear is the directionality of the movement of horse dairying, as lipid analysis of ceramics from the Eneolithic Botai culture (*c.* 3800 - 3000 BCE) is suggestive of equine milk [27], yet the horses from Botai site are from a different species than those of Bronze Age Mongolia [114]. In tracing the movement, or possibility of multiple independent origins of horse dairying future work could focus on the analysis of calculus and ceramic materials from across Eneolithic and Bronze Age Kazakhstan and central Russia for the presence of ancient horse milk proteins. In order to properly assess ancient samples for proteins from the modern and ancient equine species, we have translated published data from ancient horse genetics studies into their associated protein sequences [114, Manuscript B]. As this work is ongoing, it is vital that we expand reference protein sequence databases with possible protein sequence variations from modern and ancient animal populations, in collaboration with animal geneticists. Data produced from these upcoming analyses will further illuminate the pathways and directionality of the spread of dairy animals and practices across the steppe (Figure 11).

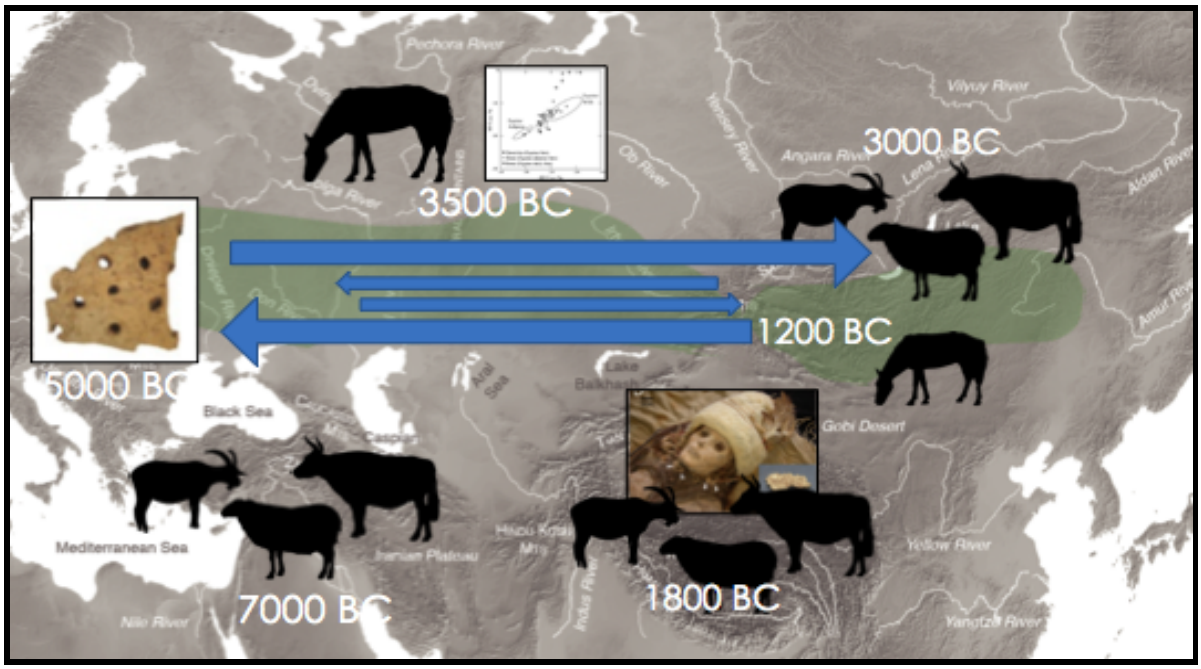

Figure 11: Map of Eurasia demonstrating the studies which bookend the evidence for dairying identified through biomolecular analyses, including lipid and protein studies, and the open questions regarding directionality and the pathways of the transmission of dairy practices.

In order to illuminate these migratory pathways, it is necessary to conduct protein studies of human dental calculus from sites between southwestern Asia and the eastern steppe spanning

between the Neolithic and Bronze Age. Prior to the advent of horse riding, early pastoralists travelled on foot and primarily lived along mountainous regions with transhumant herding patterns. Based on these observations, we have identified specific regions with similar environments, as well as other sites that contain other evidence of dairy animals and practices that present ideal populations for further study [19,22,27,114].

To expand our understanding of grain consumption in ancient and historic Mongolian diets, it would be extremely helpful to create additional datasets from faunal remains. As fauna primarily eat wild grasses and occasionally cultivated fodder, carbon values from their skeletal and dental remains offer insights into local isotopic baselines [112,122,123]. Data from fauna would be especially telling as wild plant ground cover changes dramatically across Mongolia's varied environmental zones, and factors such as aridity and temperature can affect $\delta^{13}C$ values. Furthermore, studies in migration and transhumant patterns through oxygen and strontium isotope analysis of animal and human dental enamel can also be especially telling in regards to animal herd management and the ranges of seasonal movements [124,125]. New studies using these methods across large temporal periods, such as those used in Manuscripts B and C, can uncover changes in migratory movements over time and through political and environmental changes.

6.4 Conclusion

The combination of proteomics, stable isotope analysis, archaeological, botanical, and historical evidence has allowed us to clearly demonstrate a dramatic shift from an almost purely pastoral early subsistence strategy to diversified dietary economies during the Xiongnu and Mongol Empires. We show that early Mongolian (*c*. 4000 - 800 BCE) subsistence strategies clearly included milk and meat from numerous animal species, but was also likely supplemented with hunting, gathering, and fishing. A slight, but evident increase in human stable carbon isotope values at *c*. 800 BCE demonstrates the low-level incorporation of millet into a heavily pastoral diet that included both ruminant and equine dairy. It was not until the subsequent Xiongnu and Mongol Empires (*c.* 200 BCE - 300 CE) that we see an extremely significant ($p < 0.00027$) diversification in the range of carbon values demonstrating the importance of high-input C_4 grain consumption. However, even as the Xiongnu and Mongol Empire dietary economies dramatically shifted to include locally grown and internationally traded grains, fruits, and vegetables, dairy remained economically and culturally important.

Our multidisciplinary approach has overturned previous assumptions regarding the arrival of ruminant dairying on the eastern steppe, the origins of Mongolian horse dairying, and the extreme dietary diversity of Xiongnu and Mongol empires. These datasets in these papers provide a template for future work uncovering the pathways of the dairying populations followed across the continent from Anatolia to Mongolia, revealing the extent of early horse dairying in Central Asia, and adding further resolution to our understanding of cultivated crop consumption. Furthermore, our work on understanding changes to (low- to high-investment cultivation) and the resilience of (ubiquity of dairy pastoralism through time) specific subsistence strategies in antiquity can help inform modern populations of optimal strategies for sustainable approaches to future subsistence.

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8. Summary

This dissertation explores the dietary histories of ancient Mongolia through the study of stable isotope and protein analyses, with a specific focus on the origins of dairy pastoralism and cereal grain cultivation on the eastern Eurasian steppe. In contemporary Mongolia, dairy pastoralism is a frequently practiced subsistence strategy in rural areas, and milk from as many as seven species is processed into numerous different dairy products. While dairy pastoralism and milk consumption are common in Mongolia's history, the antiquity of dairying has been unknown. The situation is similar with grain cultivation which makes up a small but important component of Mongolia's economy today, facilitated by the use of modern irrigation methods. It remains unclear, however, when grain cultivation began on the eastern steppe, and which crops were utilized. This dissertation, through three separate studies using proteomics and stable isotope analysis, aims to discover when dairy and millet were first incorporated into early Mongolian diets.

In order to better understand the antiquity of dairying on the eastern steppe, shotgun proteomics was applied to ancient human dental calculus. Through this analysis I identified the presence of ruminant (sheep or cow) milk proteins in human dental calculus at *c.* 3000 BCE from an Afanasievo-associated site in Central Mongolia. Evidence for ruminant milk consumption was also identified in the Bronze Age, Xiongnu, and Mongol Empire individuals included in this study, demonstrating the extensive and continuous history of dairy use in eastern steppe diets. Furthermore, I detected horse milk proteins in dental calculus dating to *c.* 1200 BCE, coinciding with archaeological evidence for horseback riding, indicating a dramatic shift in the economic roles of horses in the Late Bronze age. The incorporation of ridden horses into herds allowed more animals to be herded and opened the intermontane regions of the steppe for occupation and grazing. These changes may have led to an increase if population size and inter-population interactions, both of which likely impacted the formation of the subsequent Xiongnu Empire.

To refine the existing knowledge surrounding the origins of grain cultivation on the eastern steppe, analysis of stable carbon isotope ratios from human bone collagen and dental bioapatite was conducted. The results of these studies indicate the beginning of C_4 grain consumption in the Early Iron Age in north-western Mongolia, and a significant diversification of dietary intake, including grains, in the imperial regimes of Mongolia's Iron Age and Medieval periods.

These data, combined with historical documents and well-established archaeological and archaeobotanical evidence, show the extensive diversity and range of historic Mongolian diets.

This thesis identifies changes and uniformities in dietary trends in populations from over 5000 years in Mongolia. My protein data show the earliest known dairy consumption on the eastern steppe beginning at 3000 BCE, and the drinking of horse milk from 1200 BCE, both practices that continues to the modern day. I also show the slow incorporation of grain consumption into pastoral systems, through local millet cultivation, in the Early Iron Age, which increased alongside the creation of a grain-supplemented economic surplus (local and imported) during Mongolia's imperial periods. Through these studies, we can see the early and continued use of dairy in the pre-imperial periods, as well as throughout the Xiongnu and Mongol Empires, and a significant increase in the cultivation of millet through imperial-supported agriculture.

9. Zussamenfassung (Summary in German)

Diese Doktorarbeit bietet eine neue Perspektive zur Geschichte der Ernährung in der Mongolei durch die Analyse von Proteinen und stabilen Isotopen. Der Fokus besteht hierbei vor allem auf die Herkunft der Milchproduktion und der Kultivierung von Getreiden in der Ost-Eurasischen Steppe.

In der heutigen Mongolei ist die Weidewirtschaft und Milchproduktion in ländlichen Gebieten weit verbreitet und Milch von mehr als sieben Tierarten kommt in zahlreichen Produkten zur Verwendung. Obwohl diese Art der Nahrungsmittelproduktion ein wichtiger Teil der mongolischen Geschichte ist, ist wenig über die Geschichte der Milchproduktion selbst bekannt. Diese Wissenslücke betrifft auch den Getreideanbau, der zwar einen wichtigen Teil der derzeitigen mongolischen Wirtschaft ausmacht, aber dennoch weitesgehen unerforscht ist. Es noch immer unklar, wann gneau der Getreideanbau in der östlichen Steppe begann und welches die ersten Getreidearten waren. Allerdings weisen historische Dokumente aus der Zeit des Xiongnu (ca. 200 v. Chr. bis 200 n. Chr.) und des mongolischen Reiches (ca. 1250 bis 1400 n. Chr.) sowohl auf die Produktion von Milchprodukten aus der Milch von Widerkäuern und Pferden hin, als auch auf die örtliche Getreideproduktion und beschreiben sogar Verwaltungspositionen wie den ‘Vorsitzenden der Hirse-Verteilung’. Mit drei separaten Fallstudien und den wissenschaftlichen Methoden der stabilen Isotopen Analyse und der Proteomik hat diese Doktorarbeit nun das Ziel festzustellen, wann diverse Milchprodukte und der Anbau von Getreiden zum ersten Mal Teil mongolischen Ernährung wurden.

Um die Geschichte der Milchproduktion in der ostasiatischen Steppe besser zu verstehen, wurde das Konzept der Shotgun-Proteomik auf menschliche Zahnsteinproben aus archäologischen Kontexten angewendet. Durch diese Untersuchungen habe ich festgestellt, dass Milchproteine von Kühen und/oder Schafen in menschlichem Zahnstein niedergelegt wurden, der in einer auf 3000 v. Chr. datierten Fundstelle gefunden wurde, die mit der Afanasievo-Kultur im Zentrum der Mongolei verbunden wird. Beweise für den Verzehr von Milch wurden auch in Individuen gefunden, die auf die Bronzezeit datiert wurden und auch auf die Zeiten des Xiongnu und mongolischen Reiches, was auf eine umfangreiche und kontinuierliche geschichtliche Bedeutung der Milchproduktion für die Ernährung in der Steppe hinweist.

Darüber hinaus, habe ich Proteine, die in Pferdemilch zu finden sind, in menschlichem Zahnstein nachgewiesen, der auf 1200 v. Chr. datiert und mit frühesten archäologischen Hinweisen auf die intensive und vielfältige Nutzung von Pferden hinweist. Eine bedeutende Entwicklung in diesem Zusammenhang war die Aufnahme von Reitpferden in gemischte Herden, um diese zu vergrößern und mobiler zu machen. Diese Veränderungen in der Tierhaltung führten zu einem Populationsanstieg und vermehrtem Austausch zwischen verschiedenen Gruppen. Beides sind wichtige Auslöser für den Aufstieg des Xiongnu Reiches.

Um die Herkunft der Hirse in der östlichen Steppen besser zu verstehen, habe ich stabile Kohlenstoff Isotope in menschlichen Knochen und Zähnen untersucht. Die Ergebnisse dieser Studie weisen auf den Beginn von C_4-Pflanzen als Nahrungsmittel in der frühen Eisenzeit der Nord-West Mongolei hin und auf eine deutliche Diversifizierung der gängigen Nahrungsquellen zur Zeit des Mongolischen Reiches und des Mittelalters. Im Zusammenhang mit historischen Dokumenten sowie archäologischen und archäobotanischen Quellen zeigen diese Daten eine große Vielfältigkeit in der Geschichte der mongolischen Ernährung.

Diese Doktorarbeit untersucht die Veränderungen und Konstanten in den verschiedenen Ernährungsweisen der mongolischen Bevölkerung der letzten 5000 Jahre. Meine proteomischen Daten haben die frühesten Hinweise für den Verzehr von Milch in der östlichen Steppe aufgewiesen und für Kuh- und/oder Schafsmilch auf 3000 v. Chr. sowie für Pferdemilch auf 1200 v. Chr. datiert. Alle Arten von Milch sind noch heute Teil der mongolischen Ernährung. Darüber hinaus habe ich auch die sukzessive Aufnahme von Getreiden in den ländlichen Gebrauch durch die Benutzung von Hirse in der frühen Eisenzeit dargelegt, welche zusätzlich mit dem ökonomischen Überfluss zur Zeit des mongolischen Reiches angestiegen ist.

Durch diese Untersuchungen habe ich die frühen Anfänge und kontinuierliche Bedeutung der Milchproduktion aufgezeigt und den deutlichen Anstieg des Getreideanbaus als ein wichtiger Teil der Wirtschaft des mongolischen Reiches.

10. Statement of Honour (Eigenständigkeitserklärung)

I declare that I am familiar with Course of Examination for Doctoral Candidates in the Faculty of Biology and Pharmacy. I have written this dissertation myself, without using any assistance or sections of texts from third parties, or from other of my own works, without identifying them through citations or inclusions as co-authors. No one has been enlisted as a doctoral consultant, and no third parties have received any direct or indirect payment for work connected to this dissertation. This dissertation, beyond the included manuscripts that have been submitted for publication, has not be previously submitted for any other scientific examination, and none of the work presented here has been submitted to any other postsecondary school.

Jena, the 27.08.2019

Shevan Wilkin

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12. Author Contributions

Manuscript A.

"Bronze Age population dynamics and the rise of dairy pastoralism on the eastern Eurasian steppe"

Author contributions: Choongwon Jeong, Frank J. Rühli, Bruno Frohlich, Jessica Hendy and Christina Warinner designed the research plan. Choongwon Jeong, Shevan Wilkin, Tsend Amgalantugs, Abigail S. Bouwman, Richard W. Hagan, Sabri Bromage, Soninkhishig Tsolomon, Christian Trachsel, Judith Littleton, John Krigbaum, Marta Burri, Ganmaa Davaasambuu, Franziska Irmer, Erdene Myagmar, Bruno Frohlich, Jessica Hendy, and Christina Warinner performed research; Richard W. Hagan, Christian Trachsel, Jonas Grossmann, and Ashely Scott contributed new reagents/analytic tools; Nicole Boivin, Martine Robbeets, and Johannes Krause contributed personnel and material resources; Choongwon Jeong, Shevan Wilkin, William T.T. Taylor, Richard W. Hagan, Sabri Bromage, Soninkhisig Tsolomon, Christian Trachsel, Jonas Grossmann, Cheryl A. Makarewicz, John Krigbaum, Ashley Scott, Ganmaa Davaasambuu, Joshua Wright, Nicole Boivin, Martine Robbeets, Johannes Krause, Bruno Frohlich, Jessica Hendy, and Christina Warinner analyzed data; and Choongwon Jeong, Shevan Wilkin, Jessica Hendy and Christina Warinner wrote the paper. In total, Shevan Wilkin contributed to 50% of the project.

Manuscript B

"Dairy pastoralism sustained eastern Eurasian steppe populations for 5000 years"

Author contributions: Shevan Wilkin, William Taylor, Christina Warinner, Nicole Boivin, and Jessica Hendy designed the research plan; Choongwon Jeong, Shevan Wilkin, Richard W. Hagan, Christian Trachsel, Jonas Grossmann, Abigail Ramsøe, Erdene Myagmar, Jessica Hendy, and Christina Warinner performed research; Richard W. Hagan, Christian Trachsel, Jonas Grossmann, and Ashely Scott contributed analytic tools; Nicole Boivin contributed personnel and material resources; Shevan Wilkin, William T.T. Taylor, Richard W. Hagan, Christian Trachsel, Jonas Grossmann, Jessica Hendy, and Christina Warinner analyzed data;

and Shevan Wilkin and Jessica Hendy wrote the paper. In total, Shevan Wilkin contributed to 80% of this research.

Manuscript C

"Economic Diversification Supported the Growth of Nomadic Mongolian Empires"

Author contributions: Shevan Wilkin, William Taylor, Nicole Boivin, and Patrick Roberts designed the research plan; Shevan Wilkin, Alicia Ventresca Miller. Bryan K. Miller, Richard W. Hagan, Ricardo Fernandes, Madeleine Bleasdale, Erdene Myagmar, and Patrick Roberts performed research; Nicole Boivin contributed personnel and material resources; Shevan Wilkin, Alicia Ventresca Miller, Bryan K. Miller, William Taylor, Ricardo Fernandes, Richard W. Hagan, analysed data; and Shevan Wilkin, Alicia Ventresca Miller, Bryan K. Miller, and Patrick Roberts wrote the paper. In total, Shevan Wilkin contributed to 80% of this research.

13. Curriculum Vitae

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Wilkin, Shevan, William Taylor, Alicia Ventresca Miller, Bryan K. Miller, Richard Hagan, Franziska Irmer, Madeleine Bleasdale, Sumiya Gankhuyag, Christian Trachsel, Jonas Grossmann, Mark Horton, Erdene Myagmar, Nicole Boivin, Christina Warinner, Jessica Hendy. Dairy pastoralism drove Eastern Eurasian steppe expansions. (Pre-submission enquiry approved for Nature: Ecology and Evolution) — In Review

Wilkin, Shevan, Tumurbaatar Tuvshinjargal, Alicia Ventresca Miller, Bryan K. Miller, Robert N. Spengler III, William Taylor, Ricardo Fernandes, Jessica Hendy, Erdene Myagmar, Nicole Boivin, Patrick Roberts. Economic diversification, not specialized pastoralism, catalyzed nomadic empire formation in Mongolia. (Pre-submission enquiry approved for Nature: Human Behavior) — In Review

Presented talks

Food Security in Flu, Archaeological methods for economic sustainability, MPI-SHH, Department of Archaeology, Jena, Germany – "Dairy pastoralism and millet agriculture on the ancient Eurasian Steppe" — May 2019

UKAS Conference 2019, University of Manchester, UK – "Pastoralism and agriculture on the ancient Eastern Eurasian Steppe through multi-proxy archaeological data sets"

Invited talk, Botai Culture and other Neolithic Monuments of Central Asia, Al-Farabi University, Department of Archaeology, Almaty, Kazakhstan – "Using proteomics to trace dairying in ancient Eurasia" — April 2019

Invited talk, Vilnius University, Department of History – "Exploring Equine Dairying through shotgun proteomics of human dental calculus" — January 2019

Invited lecture, National Museum of Ethnology Osaka, Japan – "Processing and Consumption of Dairy Products in Ancient Eurasia: The Forefront of Milk Studies by Biomolecular Archaeology and the Connection with Northeast Asia" — December 2018

Departmental talk, MPI-SHH, Department of Archaeology – "Ancient Mongolia through proteomics, isotopes, and mummies: An update of PhD progress" — October 2018

ISBA8, Jena, Germany – "Ancient Dairying in Mongolia through LC-MS/MS bottom-up proteomics" — September 2018

EAA Conference 2018, Barcelona, Spain – "Understanding ancient Mongolian dairying through shotgun proteomics of dental calculus"	September 2018
Ancient Proteins @20, Copenhagen, Denmark – "The antiquity of dairying on the Eastern Eurasian Steppe"	August 2018
Scientific Advisory Board Lightning Talk, MPI-SHH, Jena, Germany – "Early dairy on the Eastern Eurasian Steppe through proteomics"	May 2018
Ancient Protein Workshop at the Functional Genomics Center Zurich, Switzerland – "Using LC-MS/MS methods for paleoproteomics"	January 2018
Departmental update, MPI-SHH Department of Archaeology, Jena, Germany – "Update on preliminary protein results"	September 2017
UKAS conference 2017, University College London, UK – "Pastoralism and dairying in ancient Mongolia: Insights from proteins in dental calculus"	April 2017
Eurasia Triangle invited talk, MPI-SHH Department of Linguistics and Cultural Evolution, Jena, Germany – "Early Pastoralism on the Eastern Steppe	February 2017
Departmental PhD project overview, MPI-SHH, Department of Archaeology, Jena, Germany – "Early pastoralism on the Mongolian steppe"	February 2017

Jena, the 27.08.2109

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